Myeloid-derived Suppressor Cells in Acute Myeloid Leukaemia

Athalia Rachel Pyzer

A thesis submitted for the degree of Doctor of Philosophy

Queen Mary University of London
Barts Cancer Institute
October 2016

Dedication

Dedicated to my father

Acknowledgements

The work described in this thesis was performed while I was a research fellow in the department of Bone Marrow Transplantation at Beth Israel Deaconess Medical Center, Boston USA. Six months of funding was initially provided by the British Society for Haematology and the Royal College of Physicians, UK and latterly by Professor David Avigan and the department of Bone Marrow Transplantation.

Firstly and foremost, I would like to thank my research mentor and supervisor David Avigan, for giving me the opportunity to spend the past three years in his research group and providing scientific direction, moral support and encouragement during the toughest times. More than just a boss, you welcomed me into your hospital, your community, your family and your home. I will always cherish the free reign you gave to my wildest hypotheses and the subsequent gentle nudging in the right direction.

I would like to thank my “mini-boss” and mentor, Jacalyn Rosenblatt, who expertly scientifically guided me through my PhD and welcomed me into your family and home. I’ll always remember the chicken soup deliveries when I was ill, and the many wonderful parties we had.

Thank you to my PhD supervisor, Professor John Gribben, for agreeing to take on a trans-Atlantic doctoral student and bear with me through the ups and downs. Your scientific input was immeasurably valuable and your patience with my many demands on your attention was extraordinary.

To Dina Stroopinsky, my mentor, my friend, my “lab-soul-mate”. You taught me everything I know, and I couldn’t have done a second of it without you.

There are many individuals whose help has been crucial to me in this project. They are listed below.

Kristen Palmer and Maxwell Coll taught me how to process peripheral blood and bone marrow specimens, do cell culture, western blotting and all of the necessary secret handshakes.

Vasilis Toxavidis and John Tigges in the Flow Cytometry core at BIDMC, who taught me how to do flow cytometry and provided much in the way of witty banter.

Eleni Anastasidou who taught me q-PCR technique and analysis and provided many Greek and Italian delicacies.

Nicole Pandell and John (Sean) Clohessey in the animal research facility for their expert help and support with the mouse experiments.

I must also acknowledge the patients who so generously provided blood and bone marrow samples and that have enabled this work to be carried out.

Penultimately, my fellow “close-work-colleagues”/friends in the lab and the clinic; Kristen Palmer, Maxwell Coll, Abigail Washington, Leandra Cole, Adam Ephraim, Salvia Jain, Michal Bar-Natan Zommer, Katarina Luptakova, Arie Apel, Myrna Nahas, Rebecca Karp Leaf, Hasan Rajabi, Ashujit Tagde and Poorvi Somaiya. You put up with my crazy British humour, and all my general nonsense. I love you all dearly.

Lastly, and most importantly, thank you to my family. Odette, Laurence, Gideon, Harry, Manny, Lucy, Dina, Hannah and Toby.

Abstract

The tumour microenvironment consists of an immunosuppressive niche created by the complex interactions between cancer cells and surrounding stromal cells. A critical component of this environment are myeloid-derived suppressor cells (MDSCs), a heterogeneous group of immature myeloid cells arrested at different stages of differentiation and expanded in response to a variety of tumour factors. MDSCs exert diverse effects in modulating the interactions between immune effector cells and malignant cells. An increased presence of MDSCs is associated with tumour progression, poorer outcomes, and decreased effectiveness of immunotherapeutic strategies.

In this project, we sought to quantify and characterise MDSC populations in patients with Acute Myeloid Leukaemia (AML) and delineate the mechanisms underlying their expansion. We have demonstrated that immune suppressive MDSCs are expanded in the peripheral blood and bone marrow of patients with AML. Furthermore, AML cells secrete extra-cellular vesicles (EVs) that skew the tumour microenvironment from antigen-presentation to a tumour tolerogenic environment, through the expansion of MDSCs. We then demonstrated that MDSC expansion is dependent on tumour and EV expression of the oncoproteins MUC1 and c-Myc. Furthermore, we determined that MUC1 signalling promotes c-MYC expression in a microRNA (miRNA) dependent mechanism. This observation lead us to elucidate the critical role of MUC1 in suppressing microRNA-genesis in AML, via the down-regulation of the DICER protein, a key component of miRNA processing machinery. Finally, exploiting this critical pathway, we showed that MDSCs can be targeted by MUC1 inhibition or by the use of a novel hypomethylating agent SGI-110.

These studies highlight the critical role that MDSCs play in mediating immune suppression in AML and demonstrate the clear potential for interventions targeting

MDSCs to translate to improved outcomes for patients receiving immune based therapies for their disease.

Publications

Clinical trials of dendritic cell-based cancer vaccines in hematologic malignancies

AR Pyzer, DE Avigan, J Rosenblatt

Human vaccines & immunotherapeutics 10 (11), 3125-3131

Myeloid-derived suppressor cells as effectors of immune suppression in cancer

AR Pyzer, L Cole, J Rosenblatt, DE Avigan

International journal of cancer 139 (9), 1915-1926

MUC1 Mediated Induction of Myeloid-derived Suppressor Cells in Patients with Acute Myeloid Leukaemia

AR Pyzer, D Stroopinsky, H Rajabi et al.

Blood *(In revision)*

MUC1 Inhibition Leads to Decrease in PD-L1 Levels via Up-regulation of MiRNAs

AR Pyzer*, D Stroopinsky*, J Rosenblatt et al.

Science Trans. Medicine *(Submitted)*

Abstracts

MUC1-C Inhibition Leads to Decrease in PD-L1 Levels Via up-Regulation of Micro RNAs

AR Pyzer, D Stroopinsky, J Rosenblatt, E Anastasidou et. al.

Accepted for oral presentation at the American Society of Hematology 2016

Acute Myeloid Leukaemia Cells Export c-Myc in Extracellular Vesicles Driving a Proliferation of Immune-Suppressive Myeloid-Derived Suppressor Cells

AR Pyzer, D Stroopinsky, H Rajabi, J Rosenblatt, et. al.

Accepted for poster presentation at the American Society of Hematology 2016

MUC-1 Regulates MiR34a Expression in Acute Myeloid Leukaemia Cells Resulting in an Accumulation of Granulocytic Myeloid-Derived Suppressor Cells

AR Pyzer, D Stroopinsky, H Rajabi, J Rosenblatt, MD Coll, S Jain, et.al.

Blood 126 (23), 643-643. Oral presentation at the American Society of Hematology 2015

Myeloid-Derived Suppressor Cells Are Expanded in Patients with AML and Are Dependent on MUC1 Expression

AR Pyzer, D Stroopinsky, J Rosenblatt, KA Palmer, MD Coll, M Bar-Natan, ...

Blood 124 (21), 226-226 Oral presentation at the American Society of Hematology 2014

MUC1 suppresses MiR34a expression leading to an expansion of myeloid-derived suppressor cells (MDSCs) in acute myeloid leukaemia

AR Pyzer, H Rajabi, D Stroopinsky, J Rosenblatt, K Palmer, M Coll, et.al.

British Journal of Haematology 169, 86-86

Cabozantinib eradicates advanced murine prostate cancer by activating neutrophil-mediated anti-tumour innate immunity

A Patnaik, KD Swanson, E Csizmadia, MP Gehring, K Helenius, **AR Pyzer**, et.al. Cancer Research 76 (14 Supplement), 4979-4979

Cabozantinib eradicates de novo castrate-resistant PTEN/p53 deficient murine prostate cancer via activation of neutrophil-mediated anti-tumour innate immunity

A Patnaik, K Swanson, K Helenius, T Thomas, **A Pyzer**, V Csizmadia, et.al.

Cancer Research 75 (15 Supplement), 5497-5497

Immunomodulatory Effect of SGI-110, a Novel Hypomethylating Agent in Acute Myeloid Leukaemia (AML)

D Stroopinsky, **AR Pyzer**, KA Palmer, MD Coll, M Bar-Natan, H Rajabi, et. al.

Blood 124 (21), 2303-2303

Immunomodulatory Effect of MUC1-C in Acute Myeloid Leukaemia

D Stroopinsky, H Rajabi, MD Coll, **AR Pyzer**, J Rosenblatt, S Jain, et.al.

Blood 126 (23), 3659-3659

Individual Contributions

I performed all the work described in this thesis with the following exceptions.

The mouse experiments detailed in Chapter 6 were performed in conjunction with Dina Stroopinsky and Myrna Nahas.

This work would not have been possible without the tremendous department of Bone Marrow Transplantation physicians and research nurses who obtained the consent and the samples from their patients.

Help with the identification of AML patient specimens and tabulation of AML patient data was provided by Mary-Paty Bryant.

Table of Contents

List of Figures

List of Tables

Abbreviations

MDSC Myeloid-derived Suppressor Cell

AML Acute Myeloid Leukaemia

EV Extracellular Vesicle

miRNA micro RNA

mRNA messenger RNA

TAP Transporter Associated Protein

PD-L1 Programmed Death Ligand 1

PD-1 Programmed Cell Death Protein 1

TReg Regulatory T cell

FOXP3 Forkhead Box P3

MDS Myelodysplastic Syndrome

MPN Myeloproliferative Neoplasm

FAB French:American:British

WHO World Health Organisation

APML Acute Pro-myelocytic Leukaemia

CR Complete Remission

HSCT Haematopoetic Stem Cell Transplant

GVL Graft versus Leukaemia

GVHD Graft versus Host Disease

RIC Reduced Intensity Conditioning

MAC Myelo-ablative Conditioning

CIR Cumulative Incidence of Relapse or Cytokine Induced Killer Cells

TSG Tumour Suppressor Gene

DNMT DNA methyltransferase

OS Overall Survival

HAT histone acetyltransferases

HDAC histone deacetylases

CDK1 Cyclin-dependent kinase inhibitors

IL3-RA Interleukin three receptor alpha

HSP90 Heat Shock Protein 90

IDO Indoleamine

mDC monocytic Dendritic Cell

MUC1 Mucin 1

WT1 Wilms Tumour 1

LFS Leukaemia free survival

BiTE Bispecific T-cell–engaging antibody

DART Dual affinity retargeting

CAR Chimeric Antigen Receptor

EBV Epstein Barr Virus

NOS Nitric Oxide Synthase

ROS Reactive Oxygen Species

Arg1 Arginase 1

CSFR1 Colony Stimulating Factor Receptor 1

MPO Myeloperoxidase

JAK Janus Kinase

STAT Signal transducers and activators of Transcription

TAM Tumour associated Macrophage

NK Natural Killer Cell

HPV Human Papilloma Virus

OVA Ovalbumin

ASA Acetylsalicylic acid

NAC N-Acetyl cysteine

ODN oligodeoxy-nucleotides

5FU 5-fluorouracil

PB Peripheral Blood

PBMC Peripheral Blood Mononuclear Cell

BM Bone Marrow

BMMC Bone Marrow Mononuclear Cell

DFCI Dana Farber Cancer Institute

RPMI Roswell Park Memorial Institute

Tc T cells

IFN Interferon

IL-10 Interleukin-10

TEV Tumour Extracellular Vesicle

Chapter 1 Introduction

1.1 Cancer and the Immune System

Immune surveillance

Seminal mouse experiments in the 1950s demonstrated that the immune system has the capacity to target malignant cells and destroy them. Medawar and colleagues used non-inbred strains of immune-competent mice to show that immune cells can recognise and destroy allogeneic transplanted tumours[1,2]. Moreover, mice that were lympho-depleted did not reject allografted tumours confirming the immune mediation. These discoveries led Burnet and Thomas to publish their hypothesis of cancer immunosurveillance[3,4], describing a state of frequent subclinical malignant transformation that the immune system continually surveys and destroys. In further support of immune surveillance, large scale epidemiological studies confirmed that human patients with well described immune defects had higher rates of malignancy, over and above virus-induced cancers[5]. Moreover, the advent of haematopoietic transplantation heralded the observation that allogeneic transplantation is curative for a subset of cancer patients due to the activation and expansion of alloreactive lymphocytes[6].

It was apparent that tumour cells must be phenotypically different enough from their healthy counterparts to be identified as foreign by the immune system. The answer to this question was the discovery that tumour cells have an altered repertoire of surface and intracellular proteins, or antigens, due to their malignant transformation.

Tumour antigens

Cancer cells frequently have altered expression of surface and intracellular proteins rendering them more virulent, but also a target for immune mediated destruction. These proteins, or tumour antigens, can broadly be divided into four groups. The first are antigens that are strictly tumour specific, and arise from point mutations or gene rearrangements, and can be recognized de novo by existing T cell MHC molecules. The

second are antigens normally found in male germ line cells, which do not express MHC molecules and therefore these proteins will have never been seen by T lymphocytes, and are therefore effectively tumour specific. The third category of tumour antigen are antigens normally found in healthy tissue but highly over expressed in cancerous cells, compared to their normal counterparts[7] and termed tumour-associated antigens. The final category of tumour antigen are normal antigens which undergo post translational modifications which alter them and make them phenotypically different to healthy tissue and another subset of tumour-associate antigen. An example of this is an under-glycosylated mucin, MUC1, which is over expressed by many epithelial and haematological cancers[8].

Although these alterations in the repertoire of proteins in cancer, ought to make them recognizable and targeted for destruction by effector cells of the immune system, in practice, in is rare for an established tumour to undergo spontaneous immune mediated destruction and usually only seen in highly immunogenic cancers such as melanoma[9]. Moreover, if the immune system is so finely tuned to seek out and destroy early subclinical malignancies, then why do invasive cancers occur in immune-competent people? The explanation for this is, in part, due to the many ways cancer cells avoid and alter the immune system to avert their destruction.

Cancer Immune Editing

Screiber et al have proposed "the three Es of cancer immune editing: elimination, equilibrium, and escape"[10]. The first phase "elimination", describes the original concept of cancer immune-surveillance, that is, the emerging tumour is identified as foreign by the innate and adaptive immune system and destroyed, and there is no need of progression to the subsequent phases.

The second phase "equilibrium", represents the longest phase in immune editing. In the equilibrium phase, tumour variants or sub-clones that have survived the elimination phase and the host immune system enter into a dynamic equilibrium, which can last for years. In this equilibrium, tumour cells are held in check by lymphocytes exerting

Darwinian selection pressure on the tumour bed. The end result of the equilibrium process is a new population of tumour clones with reduced immunogenicity, namely the down-regulation of tumour-associated antigens, MHC molecules and components of the IFN-Y signalling pathway[11].

Immune Escape

The last phase of immune editing, "immune escape", occurs when tumour cell variants selected for in the equilibrium phase can grow unchecked, in an immuno-competent environment, resulting in the tumour growing to become clinically detectable.

Tumours act in multiple ways to evade immune recognition. As described, tumour cells often have down-regulated expression of antigens. Furthermore, subsequent mutations and/or deletions in the tumour genome may mean that the initial tumour antigen is no longer expressed (antigen-loss variants)[12]. In addition, antigen may be presented in the relative absence of co-stimulatory molecules such as CD80 and CD86[13,14], required for the activation of effector T cells, result in a blunted T cell response.

One of the most commonly observed ways in which tumours evade the immune system is downregulating of antigen presentation machinery. Loss of MHC Class I expression, loss of individual HLA alleles and mutations of the transporter associated with antigen processing (TAP) molecules can reduce antigen presentation. Furthermore, malignant cells can inhibit the function of antigen presenting cells such as dendritic cells, by secreting molecules such as Il-10[15] and VEGF[16], inhibiting maturation and polarizing them to a tolerogenic phenotype[17]. In addition, when dendritic cells present antigen near tumour cells, it results in T cell tolerance rather than T cell priming[18], and this appears to be due to the lack of inflammation surrounding poorly immunogenic tumours. There are clinically important quantitative[19] and qualitative defects in antigen presenting cells of cancer patients. They appear of an immature phenotype as evidenced by their low expression of the co-stimulatory molecules CD80 and CD86[20,21]. Of emerging importance, plasmacytoid DCs (pDC) are a regulatory dendritic cell which

accumulate in patients with cancer, particularly around the tumour bed, and while they can effectively present antigen to T cells, they result in anergy instead of priming[22–24].

The immunosuppressive tumour micro-environment consists of increased numbers of T-regulatory cells (TRegs)[25]. These CD4+ CD25+ cells forkhead box P3 (FOXP3)+ cells produce high levels of immune suppressive Il-10. Il-10 acts on T cells and antigen-presenting cells causing anergy. It appears that numbers of circulating TRegs predict a poorer prognosis of in cancer patients[26,27]. Expansion of TRegs has been noted in patients with different solid cancers and moreover, in haematological malignancies[28,29].

Similarly to regulatory T cells, Myeloid-derived suppressor cells[30] are expanded in patients with cancer and are associated with T cell anergy and poor prognosis of disease. They will be described in further detail later.

Tumour cells may express T cell inhibitory molecules such as B7-H1[31], HLA-G[32] and HLA-E[33]. Furthermore, they may also up-regulate the CTLA-4 and PDL-1/PD-1 negative co-stimulatory pathways[34,35], which are permissive to the growth and survival of malignant cells. The importance of immune modulation in determining the balance between immune activation and disease has been highlighted by recent observations that PD-1 blockade alone is capable of inducing sustained disease response in patients with advanced solid malignancies[36]. Lastly, a variety of tumour derived soluble factors such as indolamine[37], VEGF, TGFB and IL-10 inhibit both effector T cell function and dendritic cell maturation[38].

These extensive data demonstrate the importance of a two-pronged approach in any attempt to harness the power of the immune system to eliminate tumour cells. The immune suppressive microenvironment must be reversed if any sustained potent induction of immunity against the tumour cell is desired.

1.2 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia or acute myelogenous leukaemia (AML), is a highly lethal cancer of the myeloid lineage of blood cells, characterized by the rapid growth of abnormal white blood cells in the bone marrow and peripheral blood. The bone marrow quickly becomes densely packed with leukaemic cells known as blasts, and the production of normal white blood cells, red blood cells and platelets is therefore suppressed.

Epidemiology and Aetiology

It is estimated that there are around 20,000 new cases of AML in the United States each year, the majority in adults, and roughly 10,000 deaths from AML. The mean age at diagnosis of AML is 67, and is uncommon in people less than 45 years old. The cumulative lifetime risk of a person developing AML is less than 0.5%[39].

The malignant cell in AML is a myeloblast, an immature precursor of myeloid white blood cells such as granulocytes and macrophages. In AML, a transformed myeloblast accumulates genetic mutations prevent its differentiation into mature cells. This "differentiation arrest" is combined with subsequent mutations lead to aberrant uncontrolled proliferation, defined as AML when the bone marrow infiltrate of the malignant clone reaches 20%[40].

The causes of these mutations are varied. Some are associated with pre-malignant states such as myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN), whereby accumulation of mutations due to age or exposure to carcinogens, can lead to a relatively slowly proliferating, less invasive clone which can exist for decades before transforming into invasive high grade leukaemia, and in many classes, not at all[41].

There are various chemicals and drugs that increase a person's likelihood of developing acute leukaemia, thought related to the accumulation of genetic changes. Chemotherapies such as alkylators and anthracyclines can significantly raise the risk,

with the entity of secondary leukaemia following treatment of a primary, unrelated cancer, and often many years later[42,43]. Chemicals such as benzene and organic solvents, alongside ionizing radiation can lead to acute leukaemia[44]. The development of AML is also associated with defined genetic syndromes such as Down's syndrome, or Fanconi's anemia[45,46] that have leukaemias with specific mutations linked with them.

Lastly, the majority of cases of AML are de novo, and although there are multiple mutations that are found in high frequency between patients, as genetic analysis modalities improve, the true heterogeneity of AML becomes increasingly apparent.

Classification

AML was originally classified based on the morphological appearance and cytochemistry of the blasts (French-American-British (FAB)) [47,48] but in 2001, the World Health Organisation (WHO) classification incorporated clinical features, immunophenotyping and cytogenetics into the standard morphological stratification, in order to reflect the greater heterogeneity of the disease and group diseases more effectively for more accurate prognostication[40]. The FAB and WHO classifications are summarized in Table 1.

Table 1

FAB Classification	**WHO Classification**
M0: myeloblastic, minimally differentiated M1: myeloblastic, without differentiation M2: myeloblastic, with granulocyte maturation M3: promyelocytic (APML) M4: myelomonocytic	Acute myeloid leukaemia with recurrent genetic abnormalities
	AML with myelodysplasia-related changes
	Therapy-related myeloid neoplasms
	Myeloid sarcoma
	Myeloid proliferations related to Down

M5a: monoblastic M5b: monocytic M6a: erythroleukaemia M6b: pure erythroid leukaemia M7: megakaryoblastic	syndrome Blastic plasmacytoid dendritic cell neoplasm AML not otherwise categorized • AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukaemia • Acute monoblastic and monocytic leukaemia • Acute erythroid leukaemia • Acute megakaryoblastic leukaemia • Acute basophilic leukaemia • Acute panmyelosis with myelofibrosis

Prognosis and Survival

The most important prognostic factor in AML is cytogenetic abnormality of the leukaemic cell, as summarized in Table 2. Certain cytogenetic abnormalities are associated with better outcomes (for example, the (15;17) translocation in acute promyelocytic leukaemia). Patients with “good” cytogenetics are more likely to have a remission sustained at 5 years. Roughly half of AML patients have "normal" cytogenetics and so fall into an “intermediate” risk group. The remaining patients with complex cytogenetic changes (>5 unrelated changes), -5/del(5q) or abnormalities of 3q or -7, have an “poor” outcome. They are less likely to achieve a complete remission (CR) with induction chemotherapy and they have a 78% relapse rate[49].

Table 2

Risk Category	**Abnormality**	**5-year survival**	**Relapse rate**
Good	t(8;21), t(15;17), inv(16)	70%	33%
Intermediate	Normal, +8, +21, +22, del(7q), del(9q), Abnormal 11q23, all other structural or numerical changes	48%	50%
Poor	-5, -7, del(5q), Abnormal 3q, Complex cytogenetics	15%	78%

Treatment

Given that AML is a disease of older people, it is unsurprising that half of the patients are not medically fit for aggressive treatments, and are treated with a palliative approach[50]. Younger and fitter patients usually respond well to aggressive induction therapy but have a high relapse rate due to chemo-refractory disease. Allogeneic stem cell transplantation during remission therapy significantly reduces the chance of relapse, but is unfortunately associated with a high incidence of treatment related morbidity and mortality. Novel molecular and immune based therapies that specifically target a cytogenetic aberration responsible for the malignant clone, or a protein specifically and highly expressed by the leukaemic cell, and exploit potent mechanisms of immune surveillance and elimination, offer the chance of treatment with minimal toxicity.

Chemotherapy

Treatment for AML involves induction of remission using chemotherapy, and historically total body irradiation, to induce a morphological CR defined when there are fewer than 5% leukaemic myeloblasts in a relatively normocellular bone marrow. If achieved, this is followed by courses of consolidation chemotherapy to eradicate minimal residual disease and prolong the remission. Each course of chemotherapy suppresses the bone marrow sufficient for the patient to be at high risk of neutropenic sepsis and to require multiple transfusions of packed red cells and platelets.

Historically, the mainstays of treatment for the induction of remission were the anthracycline antibiotics daunorubicin and mitoxantrone, alongside the podophyllotoxin etoposide, and the anti-metabolite cytarabine (ara-c). The combination DA (daunorubicin and ara-c) resulted in an initial complete remission (CR) rate of 60-65% when introduced and became the chemotherapy standard of care for modern chemotherapy regimens in AML[51]. The current options for front line treatment for patients it for aggressive chemotherapy, is depicted in Figure 1 below, illustrating the schedule of treatment for those patients who enrolled on the UK MRC AML-17 trial.

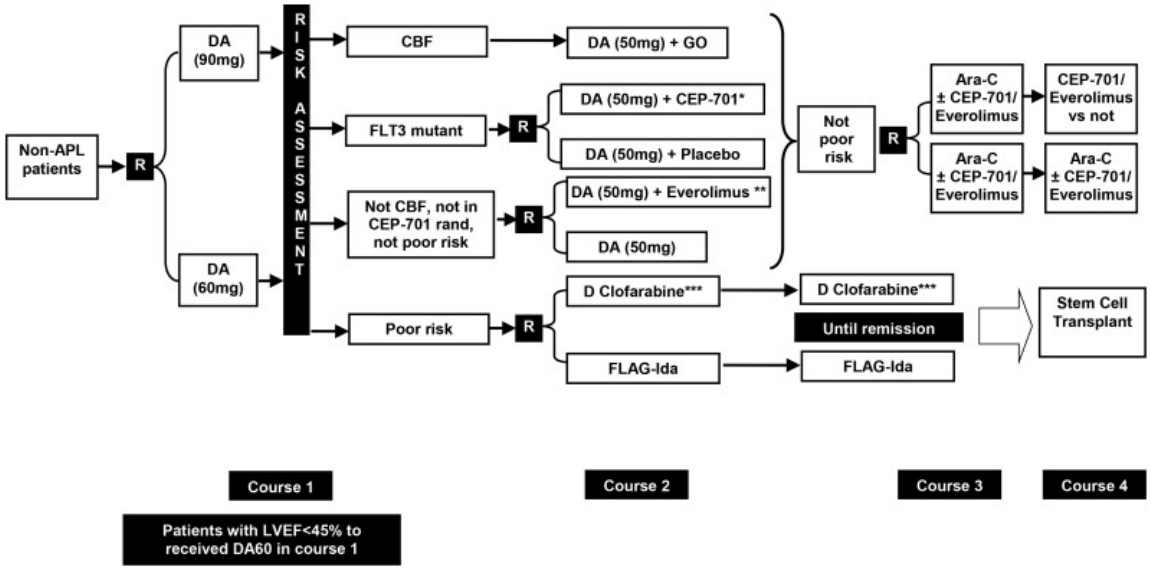


Figure 1: UK MRC AML-17 treatments for APL and non-APL (AML) treatments

Haematopoietic Stem Cell Transplant (HSCT)

The consolidation of remission by means of allogeneic transplant is routinely considered in younger AML patients with high-risk of relapsed disease. After myelo-ablation by chemo/radiotherapy, patients are given stem cells harvested from another individual, which will repopulate the bone marrow, creating a chimera. Immuno-competent donor T cells in the graft will recognize and destroy residual malignant cells, termed the graft-*versus*-leukaemia effect (GVL), as is thought responsible for the pervasive remissions seen post allograft. However, these donor T cells can also recognize healthy non-leukaemic host cells causing the life threatening complication of graft-*versus*-host disease (GVHD)[52].

Carefully designed studies examining the risk of relapse in variously risk stratified groups of patients, and comparing this to the risk of treatment related mortality, have determined that allogeneic HSCT is not recommended for patients in CR1 with a "favourable risk" profile[53] but is highly desirable in the "poor risk" group. The "intermediate risk" category poses an interesting conundrum, where despite a lack of strong evidence either way, allogeneic HSCT is generally favoured by physicians and study consortia[54].

Older patients comprise the majority of AML patients and given the prevalence of co-morbidities in this age group that may preclude traditional myelo-ablative allogeneic transplantation, new approaches have been necessary. Reduced-intensity conditioning (RIC) uses immune suppression rather than immune ablation, to allow some engraftment of donor cells, followed by repeated donor lymphocyte infusions to improve engraftment and exploit the graft vs. leukaemia effect. This results in less risk of graft vs. host disease which is life threatening to a more vulnerable older patient. Despite a lack of large scale prospective randomised controlled trial data, reduced intensity transplants now comprise 27% of allogeneic stem cell transplants in Europe[55]. Several studies retrospectively comparing myelo-ablative conditioning (MAC) and RIC

treatment in AML patients have been published. Ringden et al demonstrated that, of 434 AML patients, the roughly half that underwent RIC had lower treatment related mortality at 2 years compared with patients who received a MAC regimen (25% *v* 39%, *P* = .003). However there was a higher cumulative incidence of relapse (CIR) in the RIC compared to the MAC group (42% *v* 29%, *P* = .015) (19652066). Despite such mixed evidence, it is known that older patients who survive through any type of allogeneic transplant, in first remission, have a lower risk of relapsing compared to those not receiving a transplant (22% *v* 62%, *P* <.001), although this was tempered by an increase in treatment related mortality (21% *v* 35%, *P* <.001)[56].

In contrast to allogeneic transplantation, whereby patients are re-constituted with donor cells, the role of autologous transplantation remains unclear in the treatment of AML[57,58]. The rationale for autologous transplantation is the ability to give higher doses of chemotherapy as salvage, with the patient's own cells as rescue. However the theoretical risk of re-infusing leukaemia cells into the patient lead to efforts to purge the graft prior to infusion. The data analysing the impact of purging was inconclusive and the advent of RIC allografting and novel molecular and immune-therapeutic treatments has largely rendered autografting in AML largely obsolete.

Molecular therapy

As depicted in the schema and described in the literature, conventional cytotoxic chemotherapy agents are not the only method of induction of remission. So far, the only targeted molecular therapy to be approved for the treatment of AML was the CD33 monoclonal antibody gemtuzumab ozogamycin, which was voluntarily withdrawn from the market by the manufacturer, due to safety concerns[59]. There are, however, many therapies in the clinical trials pipeline, with varying efficacy.

Alongside the many cytogenetic mutations resulting in the translation of oncogenes or the repression of tumour-suppressor genes (*TSGs*), epigenetic changes play an important role in the pathogenesis of AML. Recent studies have demonstrated that

hypermethylation of genes is widespread in patients with AML and is implicated in the disease pathogenesis[60].

The hypomethylating agent azacitidine, a DNA methyltransferase (DNMT) inhibitor, was compared to standard of care regimens. The overall survival (OS) for patients treated with azacitidine was 10.4 months, compared to 6.5 months for patients receiving conventional care, which was not statistically significant[61]. Azacitidine's deoxy derivative, decitabine was compared to low-dose cytarabine in 485 newly diagnosed, older AML patients. The complete remission rate for the decitabine arm was 16% *vs.* 7% in the cytarabine arm, and the median overall survival was 7.7 months *vs.* 5.0 months[62].

Second-generation DNMT inhibitors have been developed such as the drug SGI-110, designed to enhance the efficacy of decitabine by combining it with deoxyguanosine. This confers resistance to degradation by cytidine deaminase, therefore increasing the half-life of the drug. A phase II trial of SGI-110 in relapsed and refractory or elderly newly diagnosed AML patients demonstrated an overall remission rate of 16% and 42.5%% respectively[63].

Histones play an important role in forming nucleosomes with DNA, and the activities of histone acetyltransferases (HAT) and histone deacetylases (HDAC) are one of the important epigenetic control mechanisms. Several clinical trials have investigated HDAC inhibitors in patients with AML with single-agent HDAC inhibitor therapy associated with response rates of 10%–20%[64,65].

Cyclin-dependent kinase inhibitors (CDKIs) are capable of inducing apoptosis in malignant cells and can also inhibit global cellular transcription. Although no CDKI is approved for use, the FDA recently granted "orphan drug" designation to alvocidib for AML, following promising results as part of induction chemotherapy in a phase II study[66].

FLT3, an often mutated receptor tyrosine kinase involved in cell signalling and proliferation, is commonly expressed on the surface of AML cells. The FLT3 inhibitor

lestaurtinib, trialled at first relapse of AML, unfortunately failed to improve OS or the CR, and was more toxic when compared to the control group[67]. Sorafenib, a multikinase inhibitor with approval in several solid cancers, is also a potent FLT3 inhibitor. Some small phase I studies of sorafenib in AML showed encouraging results[68,69], and a phase II in combination with cytarabine, demonstrated an ORR of 46% in the 37 evaluable patients[70].

The interleukin three receptor alpha (IL3RA; CD123) molecule is overexpressed in AML cells[71] and is therefore a target of treatment. SL-401, a drug comprised of human IL-3 coupled to a truncated diphtheria toxin payload that inhibits protein synthesis, was trialled in 59 patients with relapsed or refractory AML and 11 patients with new diagnoses of AML who were unfit for chemotherapy. SL-401 demonstrated single agent anti-tumour activity, was well tolerated and a randomized Phase 2b trial is planned in relapsed refractory AML[72].

The Bcl-2 family of proteins are critical to the balance between cellular survival and death. ABT-199 is a highly selective Bcl-2 antagonist that retains significant anti-tumour activity and is currently in phase II trial in AML. Notably, oblimersen, an anti-sense oligonucleotide against Bcl-2, failed to show any benefit in phase III clinical trials in combination with chemotherapy in older patients in AML[73], despite promising phase II results in combination with GO [74].

The PI3K/AKT/MTOR pathway is an intracellular signalling pathway important in regulating the cell cycle. Several drugs of interest currently in trial, which target this pathway, include AZD-5363[75], NVP-BGT226[76], OSI-027[77] and PP242[78].

Farnesyltransferase inhibitors target the protein farnesyltransferase with the downstream effect of preventing Ras protein signalling, which is commonly abnormally active in haematological malignancy[79] . The farnesyltransferase inhibitor tipifarnib[80] resulted in an overall response rate (CR + CRi + PR) of 20% in newly diagnosed AML. The addition of tipifarnib to low dose cytarabine did not, however, improve outcomes for older patients with AML in the AML-16 trial[81].

The UK's MRC AML-18 pilot trial will evaluate the safety, tolerability and feasibility of three drugs that are planned to be included in the subsequent NCRI AML18 trial. The Tyrosine Kinase Inhibitor AC220, the CXCR4 inhibitor plerixafor, or ganetespib, small molecule inhibitor of heat shock protein 90 (Hsp90), will be evaluated in combination with several different standard of care regimens of conventional cytotoxic chemotherapy.

1.3 The Immune System in AML

Historical Perspective

In 1890, an American Surgeon named William Coley observed that a cancer patient who had suffered two attacks of erysipelas had a complete remission of his tumour[82]. Coley subsequently noted that injection of solid tumours with Streptococcus resulted in tumour regression in some cases. This observation that infection can prime or tip the balance of the immune system in favour of immune mediated tumour targeting, has since been seen in several cases of AML spontaneously remitting after invasive infection[83–86]. The advent of allogeneic bone marrow transplantation in the 1960s, with its resultant potent and in some cases curative Graft vs. Disease effect[87], further demonstrated the potential of the immune system to eliminate malignant cells.

Mechanisms of Immune Dysregulation in AML

Despite these promising observations that the immune system has the capability to target and destroy cancer cells, AML cells induce a state of tolerance and immune suppression so as to evade immune surveillance.

Ineffective antigen presentation

Firstly, AML cells have down-regulated antigen presenting machinery such as MHC Class I[88] and HLA, and present in the relative abscess of T cell co-stimulation signals such as CD80[88–90].

Increased Negative Co-stimulation Signals

Negative co-stimulation and immune checkpoint signalling, are critical in immune homeostasis and their up-regulation is a key mechanism of tumour cell immune evasion. The importance of targeting inhibitory signals is key to unlocking the full potential of immune targeting on tumour cells, and is highlighted by impressive results of immune checkpoint blockade in solid tumours[91].

The PD1/PD-L1 immune checkpoint is a key inhibitory checkpoint in immune homeostasis. When tumour expressed PD-L1 binds to its receptor, PD-1, found on activated T cells, B cells, and myeloid cells, the signal is transmitted to modulate their activation. Expression of PD-L1 is seen in AML, high levels of PD1 expression on T cells predict a poorer prognosis in AML, and PD1/PD-L1 interactions have be shown to inhibit anti-tumour activity[34,92–95]. Moreover, targeting of the PD1/PD-L1 immune synapse with PD-1 blockade improves immune responses to dendritic cell based vaccination *in vitro*, and a clinical trial evaluating this combination therapy is ongoing[96].

The Tim-3/Gal-9 pathway regulates activated immune responses in leukaemia via several mechanisms. Firstly, the binding of TIM3 on exhausted T cells[97] with Galectin-9 on tumour cells, triggers apoptosis in Th1 cells. In addition, TIM3/Gal9 signalling may expand the immune suppressing MDSC population[98]. Another group has shown that TIM3/Gal9 interactions constitute an autocrine loop that is critical for the self-renewal of leukaemic stem cells[99]. Furthermore, the TIM3/Gal9 interaction may induce NK cells to produce IFNγ, which while may initially be helpful, induces IDO expression in AML blasts which in turn down-regulates NK cell activity[100]. Given these varied mechanisms of TIM3 immune suppression, it is unsurprising that the over-expression of TIM3 on T cells in AML patients has been associated with a poor prognosis[101]

Similarly to the PD-1/PD-L1 checkpoint, the CTLA-4/CD80 or CD86 interaction represents an important often up-regulated inhibitory checkpoint in many cancers. In AML, CTLA-4 is overexpressed on tumours and can be targeted to increase T cell activation[102,103].

Secretion of Inhibitory Molecules

Leukaemic blasts secrete immune suppressive inhibitory molecules in a similar fashion to innately suppressive cells such as MDSCs and TRegs. Arginase-1 production by tumour cells depletes L-Arginine, a key nutrient for lymphocytes. Arginine starvation inhibits T cell proliferation via decreased CD3 theta chain expression[104] and prevents the expression of cell cycle regulators cyclinD3 and cdk4[105] resulting in cell cycle arrest of T cells. In addition, Arginase-1 inhibits NK cell proliferation and secretion of IFN-Y suggesting widespread immune suppressive effects of this enzyme[106]. It has been shown that AML blasts produce an Arginase-1 rich environment[107,108].

Indolamine 2,3-dioxygenase (IDO) is an enzyme that catalyses the rate-limiting step in tryptophan degradation along the kynurenine pathway. Kynurenine, in turn, inhibits effector T cells and promotes regulatory T-cell (TReg) differentiation[109,110]. In immune homeostasis, IDO is produced by some alternatively activated M2 macrophages. In AML, IDO mRNA and activity in mononuclear cells was detected in 52% of patients but absent in normal subjects. Treatment of AML blasts with an IDO inhibitor 1MT, showed no significant inhibitory effect on proliferation, but a significant increase in co-cultured lymphocyte counts[111].

Regulatory T cells

Regulatory T cells (TRegs) are a population of immune suppressing cells which, in health, are a critical mediator of immune homeostasis, preventing immune over-activation and autoimmunity[112]. In patients with leukaemia, increased numbers of circulating TRegs limit anti-tumour responses[113] and predict poor prognosis[114,115]. Methods to deplete TRegs may increase NK cell mediated tumour cytolysis[116].

Natural Killer cells

Natural Killer cells, an important component of the innate immune system, and capable of recognizing and lysing tumour and virally infected cells[117], are phenotypically and

functionally abnormal in patients with leukaemia[118–120]. Furthermore, patients with AML who had lower levels of NK cell mediated tumour cytolytic activity had a poorer prognosis than their counterparts with better NK mediated tumour cell lysis, in *ex vivo* assays[121].

Dendritic Cell

Dendritic cells in leukaemia patients are quantitatively[122] and functionally deficient in presenting antigen[123]. Furthermore, there is a skew of polarity towards a tolerogenic phenotype[124] with the ratio of tolerogenic plasmacytoid Dendritic Cells (pDCs) to monocytoid Dendritic Cells (mDCs) being altered in favour of tumour tolerogenicty[125].

Mucin 1 (MUC1)

Mucin 1 (MUC1) is a heterodimeric protein that regulates critical pathways of oncogenesis including those governing cell proliferation, self-renewal, tissue invasion, and apoptosis[8]. MUC1 is aberrantly expressed in epithelial tumours and selected haematologic malignancies including multiple myeloma[126,127] and acute myeloid leukaemia[128,129]. MUC1 has been identified as a uniquely important oncoprotein in AML and AML stem cells that exerts immune-modulatory effects[8]. It has been demonstrated that MUC1 is selectively expressed on AML stem cells as compared to normal haematopoietic stem cells, and is critically involved in the self-renewal capacity of malignant cells[128].

Targeting MUC1

The MUC1 molecule is comprised of an extracellular MUC1 N-terminal subunit (MUC1-N) and a transmembrane MUC1 C-terminal subunit (MUC1-C). The MUC1-C cytoplasmic domain contains a CQC motif that is necessary for its homodimerization and its oncogenic function[130]. A cell-penetrating peptide inhibitor (GO-203) has been developed to block the MUC1-C CQC motif and inhibit MUC1-C homodimerization[131]. Treatment of

myeloma and AML cells with MUC1-C inhibitors has resulted in tumour apoptosis in *in vitro* and in xenograft models[128,132].

1.4 Immune Targeting of AML

Despite the extensive measures AML tumour cells take to evade the immune system and create a tolerogenic environment, the nature of the precarious balance between immune activation and immune suppression offers the opportunity to tip the balance in favour of re-directing the immune system to target leukaemia cells. Methods to arm the immune system to target cancer cells comprise vaccination with tumour primed antigen presenting cells such as dendritic cells, the use of immune stimulating cytokines, and more recently, the advent of engineered T cells with tumour specific activity.

1.4.1 Vaccination

Dendritic Cell Vaccines

Dendritic cells are a heterogeneous population of bone marrow derived immune cells with potent antigen presenting abilities[133–135]. Crucially, dendritic cells strongly express the co-stimulatory molecules required to induce primary immunity[134]. *Ex vivo* studies have demonstrated the ability of dendritic cells to interact with foreign antigens and present these to naïve CD4+ T cells, generating a clonal expansion of effector T cells[135]. In addition, mature dendritic cells secrete chemokines attracting B cells, resulting in the generation of a memory B cells specific to that antigen[136]. While dendritic cells in cancer patients are quantitatively[122] and functionally deficient[123], functionally potent dendritic cells can be generated from adherent peripheral blood mononuclear cells isolated from patients with malignancy by *ex vivo* by culture in the presence of cytokines. As such, dendritic cells manipulated to present tumour antigen have the potential to elicit potent anti-tumour immunity.

A variety of strategies for loading tumour antigens onto dendritic cells have been evaluated in clinical studies, including approaches that present individual peptides, protein, or whole tumour cell antigen in the context of the co-stimulatory machinery of

the DC. Previous efforts have investigated the used of (i) peptide based vaccines[137], often with an immune adjuvant[138], (ii) DNA[139–141] or RNA coding[142,143] for a specific antigen, (iii) viral/fungal vectors expressing cancer antigens[144–146] or tumour apoptotic bodies[147]. *Ex vivo* data has shown varying immunogenic responses to these techniques and some of the limitations proposed have been the need for HLA matching of peptide based approaches, potentially low immunogenicity of the chosen antigen and also the possibility that tumours could develop resistance to the vaccine by down regulating the antigen in question[148,149]. A strategy to overcome these limitations has been the use of the whole tumour cell as a source of antigen[149]. Methods for priming of DCs with whole tumour have included the whole intact tumour cell[150], cell lysate[151,152], apoptotic bodies[153,154] or whole cell DNA or RNA[151,155]. Another interesting approach has been to target antigens towards dendritic cells *in vivo*, by linking antigens to nanoparticles known to be captured by dendritic cells[156]. Our group has focused on a whole cell vaccine approach whereby patient derived tumour cells are fused to autologous, *ex vivo* generated dendritic cells by co-culture in the presence of polyethylene glycol.

Clinical trials Evaluating Dendritic Cell Vaccines in Acute Myeloid Leukaemia

An approach to introducing primed or naïve dendritic cells to patients is vaccination with a protein or peptide capable of recruiting and stimulating native dendritic cells. Several groups have conducted feasibility and phase I/II trials of peptide vaccination with peptides derived from the leukaemia associated antigen WT1, in combination with immune adjuvants[157]. Oka et al vaccinated 14 patients with AML with WT1 peptide emulsified with montanide ISA51 adjuvant at two weekly intervals. 9 of the 13 evaluable patients with leukaemia demonstrated immunological response as defined by a 1.5x increase in WT1-specific CTLs determined by tetramer assay. Moreover, this correlated strongly with clinical responses, defined as either a reduction in blast count or WT1 expression. Subsequently, Oka et al vaccinated 10 patients with AML with WT1 mRNA-electroporated dendritic cells, with an expansion in WT1 specific T cells demonstrated

and correlating with two patients in partial remission, who were converted into sustained complete remission[158,159].

A novel approach to augment antigen-presenting activity in patients with cancer has been to vaccinate them with irradiated autologous tumour cells engineered to secrete GM-CSF. It is thought that paracrine production of GM-CSF can stimulate the recruitment, maturation, and function of dendritic cells *in vivo*[160,161], overcoming the described qualitative and quantitative deficiencies of antigen presenting cells in cancer patients. Ho et al. conducted a Phase I clinical trial whereby high-risk acute myeloid leukaemia or patients with MDS were immunized with autologous, irradiated, GM-CSF-secreting tumour cells early after allogeneic, nonmyeloablative HSCT[160]. The immunization was broadly well tolerated with one patient suffering a GVHD type skin reaction. Six long-term responders showed marked decreases in the levels of soluble NKG2D ligands, indicative of NK cell tumour specific activity, and three demonstrated normalization of cytotoxic lymphocyte NKG2D expression as a function of treatment.

Vaccines using DNA/RNA encoding tumour-associated antigen:

Driessche et al. have reported their phase I/II studies of a dendritic cell vaccine made by introducing mRNA encoding the Wilm's tumour (WT1) antigen to mature dendritic cells. 10 patients with AML in at least partial remission, were treated with four bi-weekly vaccinations. Their vaccination was well tolerated and two patients who were in partial remission after chemotherapy were converted into complete remission after vaccination, and this was associated with increases in WT1-specific $CD8^+$ T cell frequencies, as demonstrated by tetramer staining[162].

Vaccines using apoptotic bodies derived from tumour cells: A recent area of interest has been the priming of dendritic cells with tumour associated antigen, in the form of apoptotic bodies. It has been postulated that apoptotic bodies allow uptake and

processing of a larger quantity of antigen, compared to dendritic cell pinocytic vesicles taking up tumour lysate or *in vitro* transcription of transfected RNA.

A similar approach to priming dendritic cells was investigated in the setting of AML. Kitawaki et al. treated four AML patients (with <20% blasts on Bone Marrow biopsy after standard of care chemotherapy), with five doses of a vaccine generated by pulsing dendritic cells with autologous apoptotic blasts, alongside KLH. Two of the four treated patients showed immune responses as determined by raised IFN levels, and the one HLA-A*2402-positive patient was shown to have had induction of CD8+T-cell responses to WT1- and human telomerase reverse transcriptase, indicating success of the dendritic cell pulsing. The two 'responder' patients appeared to have longer disease free remissions[163].

Whole tumour cell vaccines:

Our group has developed a whole cell vaccine whereby patient derived tumour cells are fused to *ex vivo* generated autologous dendritic cells. In an ongoing phase II clinical trial, AML patients who achieve remission following standard cytotoxic therapy underwent serial vaccination with DC/AML fusions. Vaccination resulted in the dramatic induction of leukaemia specific immunity, as measured by a mean fold increase of CD4 and CD8 cells expressing IFN-γ in response to *ex vivo* exposure to autologous leukaemia cell lysates and the associated expansion of T cells in the peripheral blood and bone marrow targeting the AML antigens MUC1, WT1 and Pr1. Remarkably, despite being of an average age of 61, 75% of patients (12/16) undergoing vaccination remain in sustained remission with a median follow up of 33 months[164].

Leukaemia derived dendritic cells

One approach in overcoming quantitative and qualitative defects in dendritic cells is to generate mature dendritic cells from immature myeloid cells. In AML, dendritic cells can be generated from *ex vivo* leukaemic blasts themselves, which has the added advantage of negating the need to load tumour-associated antigen, while expressing these

antigens in the context of the necessary co-stimulatory signals. A practical draw-back of this approach is that Leukaemia derived DCs can only be generated from a minority of patients, as FLT-ITD mutations or a lack of CD14 expressed on blasts prevent the maturation to the DC phenotype[165,166].

Enhancing response to Vaccination

A critical factor to consider in designing clinical trials evaluating dendritic cell vaccines is the immunologic milieu into which the vaccine is being administered. Tumour induced immune suppression may blunt immune response to vaccination in patients with advanced disease. Factors contributing to tumour induced immune suppression include an increased presence of regulatory T cells[167], increased circulating myeloid derived suppressor cells[30] and the skew of polarity of dendritic cells towards a tolerogenic phenotype[124]. Additionally, a variety of soluble factors such as indolamine, VEGF, TGFB and IL-10 inhibit both effector T cell function and dendritic cell maturation[38]. Moving forward, ongoing and future studies that incorporate vaccination in minimal disease states, following chemotherapy and following transplantation, hold promise as a means of eradicating minimal residual disease and preventing relapse. We have identified the post allograft setting as an optimal time for immunotherapy as during lymphopoietic reconstitution there is a relative depletion of immune suppressive regulatory T cells which would otherwise limit the response to vaccination[168].

A second approach toward enhancing response to vaccination is combining vaccination with stimulatory cytokines, immune-modulatory drugs, and immune checkpoint blockade.

While pre-clinical studies have used immune stimulating cytokines such as Il-2 in combination with various immune therapies, in an attempt to enhance immune responses, clinical trials have been limited to the use of GM-CSF. In the setting of AML, Borello et.al conducted a phase II trial where 28 patients were given induction chemotherapy, followed by a single immunotherapy treatment of allogeneic immortalized K562 tumour cells that had been modified to secrete high levels of GM-

CSF[169]. Patients' primed lymphocytes were collected by plasmapheresis and then underwent autologous stem cell transplantation, receiving the primed lymphocytes at day 0, followed by eight further immunotherapy treatments over a six month period. Treatment with the immunotherapy lead to a decrease in WT1 transcripts in 69% of patients after their first immune therapy, and increase in CD4+ derived IFN-gamma and granzyme. The immune therapy lead to an overall survival of 73.4% vs. 57.4% for patients who were ineligible for the immunotherapy. Of note, only six patients had detectable levels of GM-CSF at any one time point and importantly, the study was not designed to demonstrate the superiority of GMCSF modified K562 cells over wildtype.

Lenalidomide is a second-generation thalidomide analogue used in the treatment of myeloma. Lenalidomide has potent immunomodulatory functions which have not been fully elucidated but are thought to enhance activation of T and NK cells. In preclinical studies, lenalidomide enhanced immune response to vaccination[127], and a clinical trial combining lenalidomide with vaccination is planned.

The PD1/PDL1 pathway serves as a negative checkpoint for T cell activation and CTL-mediated targeting of tumour cells. In patients with cancer, up-regulated expression of PD-1 on T cell binds PDL-1 on tumour cell, resulting in the suppression of T cell capacity to secrete stimulatory cytokines[96,168]. Following autologous transplantation for myeloma, T-cell expression of PD-1 was observed to return to normal levels. In our Ex vivo study, the effect of PD-1 blockade on T-cell response to DC/whole tumour fusions was investigated. The presence the anti-PD1 antibody CT-011, promoted the vaccine-induced T-cell polarization towards an activated Th1 phenotype from a Th2 cytokine profile. Interestingly, a concomitant decrease in regulatory T cells and enhanced killing in a cytotoxicity assay was observed. A clinical trial combining PDL1 blockade with DC/tumour fusion cell vaccination for patients with AML, is underway[168].

Cytokines alone

While many pre-clinical and clinical studies have exploited the immune activating effects of particular cytokines to augment responses to other therapies, the role of cytokines as

a monotherapy remains limited. One phase III clinical trial combined the cytokines IL-2 and Histamine in the post consolidation period, to assess leukaemia-free survival (LFS). 320 AML patients were randomly assigned to treatment with HDC/IL-2 or no treatment (control). After three years, treatment with HDC/IL-2 was found to improve LFS over control, with 3-year LFS estimates of 40% (HDC/IL-2) compared with 26% (control). Side effects were typically mild to moderate, indicating that HDC/IL-2 treatment offers an efficacious and tolerable treatment for patients with AML in remission[170].

1.4.2 Adoptive T cell immunotherapy for AML

Bispecific T-cell–engaging antibodies (BiTE) antibodies, engineered antibodies capable of recognizing two antigens, have been shown to effectively recruit antigen-experienced T cells, without the requirement of pre- or co-stimulation[171]. Blinatumomab, a BiTE antibody directed at the CD19 B-cell surface antigen and the CD3e component of the T-cell receptor complex, has shown some early success in ALL[172] and based on these observations, a similar construct targeting CD33 has been developed for AML (AMG 330).

Dual affinity retargeting (DART) molecules are comprised of heavy and light chain variable domains of two antigen-binding specificities on independent polypeptide chains, which are stabilized through an engineered C-terminal bridge. A DART against CD123, which is highly and differentially expressed on AML cells compared to normal progenitor or haematopoietic stem cells, is currently tested in a phase I clinical study in relapse/refractory AML patients[173].

A highly promising immunotherapeutic strategy involves the design of T cells genetically engineered to express chimeric antigen receptors (CARs). An antibody that binds to a tumour-associated epitope is engrafted onto T cells, allowing for targeting of tumour cells triggering T cell activation and cell-mediated lysis. Efficacy is dependent on the insertion of co-stimulatory molecules such as CD28 or BB14 that promote T cell expansion and persistence in the circulation. Initial clinical results targeting CD19 in patients with CLL demonstrated durable regressions in a subset of patients with

advanced disease[174]. More recently, patients with refractory ALL have shown remarkable responses following the infusion of CD19 CARs[175]. While these dramatic successes have offered a paradigm shift for cancer therapy, the further development of this technology and its successful extension to other tumour settings is dependent on overcoming several critical issues. These include the selection of appropriate tumour antigens that effectively capture tumour heterogeneity but limit targeting of normal tissues, the identification of antibody epitopes that are close to the cell surface allowing for cell mediated lysis of the tumour target, an ongoing source of co-stimulation to facilitate T cell expansion and memory responses, and the opportunity for epitope spread allowing for the targeting of additional tumour antigens.

One of the first attempts to utilize CAR technology for AML was using CAR transduction into cytokine-induced killer (CIK) cells. An Italian group showed than an anti-CD33 CAR was able to enhance the anti-leukaemic function of CIK cells, in *ex vivo* assays[176]. Furthermore, they showed that their anti-CD33 CAR also redirected Epstein-Barr virus (EBV)-specific T cells towards human $CD33^{+}$ cells in a murine model of AML[177]. As with BiTE molecules, a CAR against CD123 was developed and demonstrated to be superior to the anti-CD33 CAR in safety and efficacy, in murine models[71].

In terms of ongoing clinical trials using CARS, an anti-CD33 CAR is in trial for relapsed or chemo-refractory AML patients, with one patient having been reported to have a dramatic, if short lived, reduction in blast count[178]. In addition, a trial of anti CD123 CAR construct has been opened for relapsed or refractory AML patients[179].

1.5 Myeloid-derived Suppressor Cells

1.5.1 Historical Overview

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells with immune suppressing capability, that play a critical role in creating the immunosuppressive milieu of the tumour microenvironment[30].

While the notion of bone marrow cells with immune-suppressing capabilities had been described as early as 1979[180], the first lineage-defined population of immature myeloid cells in mice with immune-suppressing effects was described in the mid-1990s by Bronte et al[181] who reported a population of splenocytes which inhibited CD8 T cell responses to viral immunogens. Subsequent studies in tumour-bearing mice identified CD11b and Gr+[182–184] as markers defining this population. Inducible Nitric Oxide Synthase (NOS)[185], Reactive Oxygen Species (ROS)[186] and Arginase-1 (Arg-1)[187] were elucidated as mediators of immature myeloid cell T cell suppression[182,188]. Concurrent studies confirmed a similar population of cells in humans[31,189–191]. Gabrilovich et al coined the term *Myeloid-derived Suppressor Cells* (MDSCs) [192] to describe this heterogeneous mixture of myeloid cells with an immature phenotype and expanded in response to a variety of tumour-derived cytokines [30].

During the last decade, it has been shown that MDSCs exert diverse effects in modulating the interactions between immune effector cells and the malignant cells. Increased presence of MDSCs is associated with pro-tumoural processes such as angiogenesis[193,194], tumour progression[195], metastatic spread[196] and poorer outcomes[197]. Moreover, MDSCs have been demonstrated to reduce the effectiveness of immunotherapeutic strategies[198]. Efforts to target MDSCs to maximize responses to cytotoxic and immune based treatments for cancer, is currently a major area of investigation [199].

1.5.2 Mouse MDSCs

In mice, MDSCs co-express the myeloid marker CD11b and the granulocyte marker Gr-1. The Gr-1 antibody binds two isoforms of the Ly6 molecule, Ly-6G, which is expressed on the granulocytic subset of murine MDSCs, and Ly-6C which is highly expressed on monocytic phenotype MDSCs, and weakly on granulocytic MDSCs[200].

Under physiological conditions, mice bone marrow, unlike in humans, contains a significant proportion of Gr1+ CD11b+ cells, ranging from 12%[201] to 60%[202] in different strains. Only a subset of these cells will be true MDSCs, with the capacity to suppress immune responses, reinforcing both the need to define MDSCs as cells with an immature myeloid phenotype and immune suppressive function, and the need to elucidate more specific markers for these cells.

1.5.3 Characterization of murine MDSCs

There have been several other proposed markers for murine MDSCs, but all lack high sensitivity or specificity. The integrin subunit CD49d has been reported as a marker for monocytic MDSCs in mice, differentiation suppressive from non-suppressive cells[203], although another study disputed this separation[204].

The suppressive activity of monocytic MDSCs correlated with the expression of the macrophage colony-stimulating factor receptor CD115 (CSF-1R), in murine models of colon cancer[205] and melanoma[206]. However CD115 is also expressed on monocytes, macrophages, osteoclasts, as well as on common dendritic cell precursors and macrophage/dendritic cell precursors, rendering its solo specificity low.

In a murine model of lymphoma, CD244 (commonly found on Natural Killer cells and some T cells) expression distinguished granulocytic MDSCs from neutrophils, while CD124 (IL-4Ralpha) and the inflammatory proteins S100A8 and S100A9 did not[207].

Several studies have noted that in murine models of solid cancers, both monocytic and granulocytic MDSCs up-regulate expression of the macrophage marker F4/80[208] as they enter tumour microenvironment, on their way to differentiation into CD68+ Tumour associated Macrophages. As such, F4/80 might be useful as a maker of a more mature MDSC or tumour infiltrating MDSC[209].

The myeloid pro-inflammatory proteins S100A8 and S100A9 were shown to be highly expressed on murine MDSCs and involved in their expansion in a murine model of colon cancer[210].

As described, both subsets of murine MDSCs can be induced in a diverse array of murine models of cancer[209,211] and exert similar suppressive effects to human MDSCs. It is notable that monocytic murine MDSCs are thought to be more suppressive than their granulocytic counterparts, predominantly exerting their effects via the production of Arginase-1 and iNOS, whereas granulocytic MDSCs produce immune suppressive ROS, alongside Arg-1 and myeloperoxidase (MPO)[208,209,212].

1.5.4 Human MDSCs

In humans, MDSCs are generally characterized by the expression of the myeloid markers CD11b, CD33 and low or absent HLA DR. Monocytic and granulocytic subsets of MDSCs have been identified characterized by expression of CD14 and CD15, respectively[30].

While both subtypes are present at low levels in healthy subjects [213], they are ubiquitously expanded in patients with cancer[30], and various inflammatory[214,215] and pre-malignant conditions[216].

In addition to the granulocytic and monocytic subtypes, a third subset of putative MDSCs with the phenotype Lin-/HLADR-/CD33+/CD11b+/CD14- and a promyelocytic appearance were described in breast cancer patients[217] and inversely correlated with clinical response. These cells, perhaps representing early granulocytic MDSCs, highlight an increasing understanding of the heterogeneity of this population. Furthermore, these populations may exist in a dynamic state of differentiation, with emerging evidence that monocytic MDSC may differentiate into granulocytic[218].

There has been much effort to identify more specific MDSC markers, as myeloid subtypes share common markers[219], with all of the common used “MDSC markers” (CD33, CD11b, CD14 and CD15) being variably expressed on other myeloid cells.

The granulocyte marker CD66b is commonly but not ubiquitously expressed on granulocytic MDSCs in humans, and can be used in lieu of CD15, but is of course also expressed by "non-MDSC" granulocytes. VEGFR1 was reportedly highly expressed on granulocytic[220] MDSCs in humans, although another study reportedly lower levels[221].

CD124, also known as the alpha-chain of the receptor for IL-4 (IL4Ralpha), was demonstrated to be expressed on suppressive polymorphonuclear cells and was critical for their negative activity on CD8 T cells[222], but is also expressed on mature T cells, B cells and monocytes.

Following revealing studies in mice[210] the myeloid pro-inflammatory protein S100A9, and to a lesser degree its closely related proteins S100A8 and S100A12, were demonstrated to be 10-fold to 15-fold higher expressed in MDSC than in monocytes and strongly associated with immune suppressive NOS expression[223], and in another study in NSCLC, Arginase-1 expression[223], NOS and Arginase-1 production being hallmark features of MDSCs. Furthermore, a study in gastric cancer showed levels of circulating S100A8 and S100A9 were increased compared to healthy controls, and correlated with MDSC numbers[224]. However S100A8 and S100A9 are found in wider populations of granulocytes, monocytes and the early differentiation stages of macrophages. A functional limitation of using S100 family members to phenotype cells is that they are all intracellular proteins, limiting their use in functional studies where live cells need to be retained[225]. Building on these observations, Qin et al identified two phage eluted peptides corresponding to parts of, and co-immunoprecipitating with S100A8 and S100A9, and were able to partially deplete both granulocytic and monocytic MDSCs in a murine model, resulting in inhibition of tumour growth[226]. However there was some off target depletion of NK cells in this model, reminding us that currently, there is no marker that is 100% sensitive and specific for MDSCs. We speculate that while it remains possible that a specific MDSC marker will be discovered, the perhaps more likely outcome of these studies will be that the lack of specific markers represents the idea that MDSCs are a group of immensely disparate cells with a common suppressive

phenotype, and therefore that functional markers, such as indoleamine and Arginase-1 (discussed below) may be a better way of characterizing this group of cells, and specifically targeting them *in vivo*.

1.5.5 Technical issues in defining human MDSCs

Separation from peripheral blood

While some studies have defined MDSCs as a fraction of whole blood, most have used PBMCs following gradient centrifugation. The rationale for this is that whole blood includes a large population of polymorphonuclear cells, phenotypically indiscriminate from granulocytic MDSCs. When whole blood is analysed, higher density PMNs which are excluded from gradient centrifugated PBMCs, showed no suppressive activities, compared to lower density PMN cells – the population that includes granulocytic MDSCs[227], justifying the continued use of techniques such as ficoll density centrifugation.

Cryopreservation

Several studies have investigated the effect of cryopreservation on the quantity and function of MDSCs, the consensus being that frozen samples retain their monocytic fraction of MDSCs but granulocytic MDSCs do not survive the thawing process[228–230]. Moreover, both subtypes lost their immune suppressing effects on T cells after cryopreservation and thawing[230]. In conclusion, until better methods of freezing and thawing cells are developed and validated, studies involving MDSCs should be performed on fresh samples.

1.5.6 The Expansion and Activation of MDSCs

Myeloid differentiation from haematopoietic stem cells is characterized by the emergence of functionally potent cell populations including dendritic cells, granulocytes

and macrophages[231]. In the setting of chronic infection, inflammation, trauma or malignancy, the associated cytokine milieu favours abnormal accumulation of immature myeloid cells that manifest an immune-suppressive phenotype [30]. This likely represents a compensatory response to chronic immune stimulation preventing the over-stimulation of immune effector cells that can result in by-stander damage[232]. In malignancy, however, this alteration in the immunologic milieu is utilized to facilitate promotion of tumour growth[233], and dissemination[234], the immune-paresis of malignant disease[235], and limiting response to immune-based therapies[236].

MDSC expansion has been described in many cancer models including Renal Carcinoma[220]; Melanoma[237]; Prostate[238]; Hepatocellular Carcinoma[239]; Head and neck cancer[240]; Rectal Cancer[92]; Colon and Breast Carcinoma[241]; Glioma[242]; Pancreatic[243] and Non-small cell lung cancer[244].

1.5.7 MDSCs in Haematologic Malignancies

More recently several studies have investigated the role of MDSCs in haematological malignancies, demonstrating their importance in lymphomas such as B-cell non-Hodgkin lymphoma[245,246] and Hodgkin's Lymphoma[247] and Chronic Lymphocytic Leukaemia [248,249].

In Multiple Myeloma, several studies have suggested the expansion of MDSCs[201,250–252]. In a recent report, MDSCs (CD11b+CD14-HLA-DR-/lowCD33+CD15+) were expanded in the peripheral blood and the bone marrow of patients with multiple myeloma as compared to healthy controls. MDSCs were shown to supported tumour cell growth while simultaneously suppressing T-cell–mediated immune responses[253].

In myeloid malignancies, where the tumour cell phenotypically resembles an immature myeloid cell, the differentiation of an MDSC from a tumour cell poses some difficulty. In one study in CML[254], investigators attempted to differentiate the tumour clone by the presence of CD34 positivity, whilst noting that 35% of MDSCs expressed CD34, indicating a high likelihood of misidentification.

While increased numbers of MDSCs have been suggested in patients with Myelodysplastic syndrome, with concurrent increases in MDSC derived TGF-β, VEGF and IL-10[216], there has been little published about MDSC populations or their function in AML. Of note, immature myeloid cells such as MDSCs share common characteristics with myeloid leukaemia cells due to the early maturation arrest of leukaemic cells. For example, it has been suggested that AML blasts exert their suppressive effects on T cells via a similar Arginase-1 dependent mechanism to MDSCs[255].

1.5.8 Factors Involved in the Expansion of MDSCs in Cancer

Tumour Secreted Factors Involved in MDSC Expansion

A series of murine and human *in vitro* and *ex vivo* studies suggest that tumour cells secrete factors into the microenvironment that promote MDSC expansion.

In human studies, tumour-conditioned medium has been used to expand MDSC-like cells from peripheral blood mononuclear cells[256] with GM-CSF an IL-6 proving critical mediators of this expansion.

In murine studies, tumour-conditioned medium expanded MDSC-like cells from bone marrow mononuclear cells[257] or splenocytes[258], with GM-CSF being repeatedly reported to be the key inflammatory mediator[259–261]. Other implicated cytokines in murine MDSC expansion include M-CSF[262], G-CSF[263], SCF[264], CSF[265], IL-6[266], Il-1B[267] and TNFalpha[268].

Extracellular vesicles (EVs) are membrane bound vesicles released ubiquitously by cells and are thought to be important mediators of inter-cellular communication. EVs have a complex nomenclature, which includes the terms exosomes, microvesicles and oncosomes, defined by size, and ranging from 40-1000nM[269–271]. While their biological relevance in cancer has yet to be fully elucidated, it is generally agreed that they carry biologically relevant proteins, mRNAs and microRNAs[271]. In a murine *in vitro* model of Breast cancer, tumour secreted exosomes contained PGE-2 and TFG-B which led to an expansion of MDSCs[272]. In an human *ex vivo* model of melanoma, melanoma cell line secreted micro-vesicles lead to an expansion of MDSCs [273].

Key signalling pathways Involved in MDSC Expansion

The Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway has a critical role in mediating both the expansion of MDSCs and their function in suppressing immune cells[274–276]. Unsurprisingly, inhibitors of STAT activation have been used to attempt to target this population, as we will later discuss. STAT3[277] and STAT5 have key roles in MDSC expansion, putatively via their roles in inflammatory cytokine production[278]. STAT3 inhibition has been shown to mediate differentiation of MDSC into mature Dendritic Cells, indicating aberrant STAT3 signalling plays an important role in cancer, in maintaining myeloid cells in an immature, more immune-suppressive state[274,279]. In head and neck cancers STAT3 was shown to control MDSC function by regulating Arg-1 activity[280]. MDSCs from tumour bearing mice have high levels of activated STAT3[281]. The inhibition of STAT3 with the tyrosine kinase inhibitor sunitinib, blocked expansion of MDSC in tumour bearing mice[282]. Consistent with this finding, activation and overexpression of STAT3 in myeloid cells led to an expansion of MDSC in murine model of lung cancer[283]. STAT3 activation up-regulated the pro-inflammatory protein S100A8/9, which inhibits the differentiation of DCs and macrophages, leading to an accumulation of MDSCs[210]. There is an inverse correlation between the percentage of granulocytic MDSCs and levels of STAT1 phosphorylation in CD4 T cells. Co-culture of

MDSCs and CD4 T cells from healthy donors led to reduced IFN-y responsiveness[284]. STAT6-deficient MDSCs fail to inhibit T cell activation as they failed to up-regulate iNOS or make Arg-1[285].

The transcription factor Twist is associated with diverse malignancies. In one study, overexpression of Twist in cancer cell lines was associated with an expansion of murine MDSCs in co-cultured myeloid precursor cells. Conversely, siRNA silencing of Twist expression partly abrogated the expansion of MDSCs in this model[286].

The RAS signalling pathways are key regulators of normal cell growth and malignant transformation[287]. Ras signalling plays a key role in myeloid development and promotes granulopoeisis, and thereby the production of granulocytic MDSCs, by increasing the binding of C/EBP alpha to the GCSF receptor[288]. Overexpression of kRas in a murine model of pancreatic cancer cells led to increased expression of the chemoattractant cytokines MIP-2 and MCP-1, which promote the recruitment of macrophages and MDSCs into the tumour microenvironment[289].

PI3K/Akt signalling affects cell growth, survival migration and metabolism that is thought to play a significant role in MDSC expansion. Aging mice accumulate MDSCs in their BM and secondary lymphoid organs which was demonstrated to be related to a PI3K/AKT signalling defect in MDSCs [290]. Moreover, SHIP and PTEN proteins are negative regulators of PI3K signalling[291] and a dramatic increase in MDSCs is seen in SHIP knock out mice[292].

MicroRNAs Involved in MDSC Expansion

An alternate mediator of oncogenesis is the presence of microRNAs that bind and degrade specific mRNAs disrupting translation and creating post-transcriptional effects that promote the malignant phenotype.

In one study, the microRNAs miR155 and miR21 were the two most upregulated miRNAs during the induction of MDSC from the bone marrow cells by GM-CSF and IL-6 and

targeting these miRNAs abrogated cytokine induced MDSC expansion *in vitro*[293]. miR155 was further demonstrated to mediate tumour induced MDSCs in a murine models of lymphoma[294], and breast cancer[295].

MiR34a targets p53 and has been linked to the expansion of MDSCs. The combination of Twist silencing and miR34a overexpression nearly completely abrogated the expansion of MDSCs[286].

Other Factors Promoting MDSC Expansion

Hypoxia is a common feature of solid tumours as they outgrow their blood supply[296], and has implicated in mediating resistance to chemotherapy[297,298], the promotion of metastases[299], as well as resulting in an immune privileged niche[93,300–302]. Hypoxia has been shown to promote the expansion of immune-suppressive MDSCs[303,304], as well as mediating the differentiation of MDSCs into immune-suppressive tumour associated macrophages (TAMs), upon arrival in the hypoxic tumour bed[302].

While the presence of tumours has been clearly demonstrated to result in the recruitment and proliferation of MDSCs, various cancer treatments have also been implicated in further expanding this population. Several cytotoxic chemotherapeutic agents including cyclophosphamide[305,306], doxorubicin[234] and melphalan have been associated with an expansion of MDSC-like cells, although in one study these "induced MDSC" were not as immune-suppressive as native MDSC[307]. It is worth noting that GM-CSF, widely used as an immune adjuvant in cancer vaccines, is the most reported MDSC promoting cytokine, although evidence of MDSC expansion in GM-CSF containing treatments remains conflicting[308].

While we have divided MDSC inducing factors into discrete sections, it is important to note that there is a huge amount of cross-talk and overlap in these pathways, with transcription factors mediating cytokine release, and miRNAs, such as miR155, implicated in promoting transcription factor STAT3 activity[277].

1.5.9 Mechanisms of MDSC-mediated Immune Suppression

MDSCs inhibit the function of effector and antigen presenting cells in the tumour microenvironment prevention the effective activation of tumour specific immunity.

MDSCs and T cells

A critical mechanism by which MDSCS directly induce lymphocyte suppression is L-Arginine depletion, a key nutrient for lymphocytes. Arginase-1 catabolizes L-Arginine to urea and ornithine[309,310]. Arginine starvation inhibits T cell proliferation through decreased CD3 theta chain expression[104] and prevents the expression of cell cycle regulators cyclinD3 and cdk4[105], resulting in cell cycle arrest of T cells. In addition, Arginase-1 inhibits NK cell proliferation and secretion of IFN-Y, suggesting a broader immune-suppressive effect on effector cell function [106]. Furthermore, treatment with the Arginase inhibitor nor-NOHA delayed the growth of tumours in an immune-competent murine model of lung cancer[104], highlighting the importance of this pathway in regulating tumour growth. Of note only granulocytic MDSCs produce high levels of Arginase-1 in humans, whereas in mice both monocytic and granulocytic fractions demonstrate this capacity [311].

MDSCs generate oxidative stress by increasing levels of ROS and iNOS with resultant immunosuppressive effects. ROS and iNOS activity lead to the production of reactive nitrogen species such as perioxynitrite, H202 and NO[309,310]. NO suppresses T cell function via the Jak/Stat signalling pathway[312], reducing MHC expression[313], inducing T cell apoptosis[314], promoting the loss of theta expression[315] and the nitration and desensitization of the TCR[316]. This mechanism has been demonstrated in patients with pancreatic cancer whose granulocytic MDSCs reduced CD3 theta chain expression via an H_2O_2-dependent mechanism[315]. Furthermore, NOS inhibitors reversed MDSC induced immunosuppression *in vitro* and *in vivo* models of cancer[310,317]. In humans, granulocytic

MDSC produce more ROS than monocytic MDSC[207], who act primarily via iNOS production[318].

Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme known to polarize antigen-presenting cells towards a tolerizing phenotype. In CLL, monocytic MDSCs express high levels of IDO. Conversely, blocking IDO in this setting resulted in an increase in T-cell proliferation and a decrease in regulatory T cell induction[249]. Similar findings were reported in the post-allograft setting, where monocytic MDSCs exert an immunosuppressive effect via IDO production[319].

The PD1/PD-L1 signalling pathway is a critical mediator of immune tolerance in the tumour microenvironment. MDSC express the immune inhibitory ligand, PD-L1, ([93]) which engages PD-1 on T cells resulting in an exhausted phenotype. Similarly, MDSCs express Galectin 3, the ligand for TIM-3 on lymphocytes, capable of inducing T cell apoptosis[320]. PD-L1 signalling was important to the immune suppressive activity of MDSCs in mouse models of several cancer types[93] and delivery of a lentiviral vector carrying shRNA against PD-L1 abrogated MDSC immune suppression in a murine *ex vivo* model of melanoma[321]. However, the significance of PD-L1 expression on human MDSCs remains ambiguous. In one example, patients with melanoma were found to have circulating monocytes with a suppressive phenotype and high levels of PD-L1 expression, inversely correlated with HLA-DR expression and suggestive of MDSC phenotype[322]. However in patients with glioma, PD-L1 could not be detected in any subset of MDSC[242].

TGF-beta is produced by immature myeloid Cells from tumour bearing mice that, in conjunction with NO, inhibits T cell proliferation *in vitro*[323]. Blocking TFG-beta or depleting MDSCs prevented tumour recurrence showing that TGF-beta production is crucial to their immune suppressive activity[324]. TGF-beta, in turn, induces MDSC proliferation by promoting MDSC miR494 expression[325]. Furthermore, blocking exosomal TGF-beta reduced MDSC induction[272]. In addition, MDSC can inhibit immune

effector responses in a contact dependent manner via membrane bound TFG-beta which can induce anergy in NK cells[326].

The prostanoid PGE2 has both pro-inflammatory and immune-suppressive properties and is synthesized by COX2. PGE2, induced by tumour-derived factors[327], signals through PGE2 receptor E-prostanoid 4 which induces Arginase 1 production in MDSCs[328,329]. *In vivo* administration of COX2 inhibitors reduced MDSC accumulation in lung cancer bearing mice[328].

Tumour-derived IL-1B has been shown to induce the accumulation of MDSCs[267] but it is also important to their activation. IL-1B stimulation of MDSCs led to increased NFKB activity leading to an increase in suppressive activity[330], putatively via the production of Indoleamine[331].

Preclinical models have demonstrated MDSCs mediate antigen specific inhibition of CD8 T cells in the setting of malignancy. MDSCs were shown to specifically induce T cell tolerance against peptides presented by MHC class I on the MDSC [332]. This studies suggest antigen-specific T cell tolerance requires direct cell contact and is mediated by the production of ROS[333]. However the presence and mechanism of CD4 T cell suppression remains less clear. While some groups have reported MDSC-induced CD4 T cell tolerance[285,334,335], other studies have failed to demonstrate this [186,336]. More recently, Nagaraj et al demonstrated that MDSCs can indeed induce CD4 T cell tolerance via MHC class II expression, however in tumour bearing mice, MHC class II is down-regulated compared to healthy mice[337]. This down-regulation of MHC Class II, putatively via STAT 3 signalling[338], may explain some of the conflicting data regarding CD4 T cell immune suppression.

MDSCs and Regulatory T cells (TRegs)

Regulatory T cells (TRegs) are a critical component of tumour-related immune suppression subject to regulatory influences by MDSCs. TRegs are recruited by MDSC production of TGF-B and IL-10[205] and through CD40-CD40L interactions[239]. MDSCs generated by GMCSF in mice, were found to delay skin allograft rejection in a TReg generating mechanism, raising the potential for MDSCs to be used in the non-cancer setting[339]. One group has succinctly demonstrated that FOXP3 positive TRegs could be induced from Th17cells by MDSC-derived TGF-beta and retinoic acid[340]. Furthermore, *in vitro* and *in vivo* inhibition of MDSC activity with the Arginase-I inhibitor NOHA or Sildenafil abrogated TReg proliferation[341]. MDSCs from patients with hepatocellular carcinoma were found to stimulate IL-10 producing TRegs in an *ex vivo* experimental setting[239].

MDSC and Natural Killer (NK) Cells

NK cells are critical effector of innate immunity with the capacity to target and eliminate malignant cells. NK cell function is governed by inhibitory and activating receptors that may be selectively engaged dependent on the microenvironment in which they reside. MDSCs from patients with hepatocellular carcinoma inhibited autologous NK cell cytotoxicity and IFN-Y in an *in vitro* model. This suppression was dependent on cell contact and relied on the NKp30 ligand on NK cells[342]. Moreover, there appears to be a feedback mechanism between MDSCs and NK cells, with NK cells regulating MDSC expansion[246].

MDSCs and Dendritic Cells (DCs)

Dendritic cells (DCs) represent a complex network of activating and tolerizing antigen presenting cells that include myeloid derived cells that are uniquely capable of inducing primary immune responses. DC maturation is associated with the up-regulation of co-stimulatory molecules and potency of antigen presentation. As MDSCs accumulate in models of cancer, the population of mature dendritic cells reduces

proportionately[210,343], in keeping with the hypothesis that tumour derived factors place a differentiation block on myeloid differentiation. Indeed, under the right conditions, MDSCs may be forcibly differentiated into dendritic cells[344].

MDSCs inhibit DC function by producing IL-10, which inhibits DC TLR-induced IL12, reducing DC mediated activation of T cells[345]. Furthermore, MDSCs hinder DC based immune therapy, one group demonstrating that DC generated in the presence of MDSCs are deficient in antigen uptake[346].

MDSCs and Macrophages

MDSCs are strong producers of IL-10, which acts on macrophages which, in turn, produce IL-10 and are predisposed to Th2 reactions[347]. It appears there is a bi-directional model of cross talk between MDSCs and macrophages, with macrophages themselves stimulating MDSCs to produce more IL-10[347]. Furthermore, MDSCs recruited into the tumour bed may become differentiated into tumour-associated macrophages (TAMs), immune suppressive macrophages[344,348], as evidenced by an up-regulation of CD68.

1.5.10 Pre-clinical Studies

Investigators have examined strategies to deplete MDSC expansion, limit recruitment or inhibit their function to enhance tumour specific immunity and effectively target malignant cells alone or in combination with other immunotherapeutic strategies.

Inhibition of MDSC recruitment to the tumour microenvironment has been attempted by targeting the Colony Stimulating Factor Receptor 1 (CSF1R) through inhibition[349] or receptor blockade[350], or by targeting the chemokine CCL2[351] and the CXCR4/CXCL12[352] axis, with a reduction in tumour growth in multiple murine models of cancer (see Table 3).

Gemcitabine depletion of MDSCs in a murine model of lung cancer, improved responses to IFN-beta treatment, attributed to a surge in NK cell mediated tumour cytotoxicity[353].

Similar effects of Gemcitabine treatment were seen in a murine model of breast cancer, where mice treated with a HER-2/neu vaccine, anti-GITR antibodies and Gemcitabine had stronger anti-tumour immunity compared with non- Gemcitabine treated mice[354].

ATRA was successfully used to deplete MDSCs in murine models of adeno- and fibro-sarcoma treated with a C3-peptide vaccine or p53-based vaccine, with ATRA-treated mice showing improved CD4 and CD8 T cell mediated tumour-specific immune responses and prolonged anti-tumour responses[355].

In a murine study, mice treated with sunitinib demonstrated depletion of MDSCs and associated levels of TRegs resulting improved anti-tumour immunological responses[356]. The addition of sunitinib to vaccine against human papillomavirus (HPV)-induced cancer, demonstrated increased survival mirrored by encouraging immunological responses, compared to vaccine alone[236]. In a murine model of melanoma, ovalbumin (OVA) peptide-pulsed dendritic cell vaccine was given to mice, with or without sunitinib treatment. In a third murine study of sunitinib, lung carcinoma bearing mice were treated with intra-tumoural IL-12 gene delivery by adenoviral vector + 4-1BB activation, with or without sunitinib. Mice treated with sunitinib had fewer MDSCs and TRegs, but moreover, the expression of negative co-stimulatory molecules CTLA4 and PD-1 in T cells and PDL-1 expression on MDSC and dendritic cells, was also significantly decreased. These immunological parameters were also associated with improved outcome to the immunotherapy[357].

Adoptive immunotherapy using T cells genetically modified to express antigen-specific chimeric antigen receptors (CAR) is a promising development for the treatment of cancers[358]. MDSCs have been implicated in limiting the response to CAR-T cell therapy, in murine models of osteosarcoma[359] and liver cancer[360]. Moreover, depleting MDSCs improved anti-tumour efficacy in both models. Furthermore, in a murine model of breast cancer, a combination treatment of anti-Her2 CAR-T cells and PD-1 blockade had a depleting effect on MDSCs, although this may be purely an effect of a reduction in

tumour burden or, as the authors suggest, a direct effect of PD-1 blockade on small subset of MDSCs that expressed PD-L1[361]. In support of this argument, an antibody against PD-L1 in colon cancer bearing mice lead to a reduction in MDSC number, in an FC-receptor dependent manner, or independent of tumour burden[362].

Table 3 summarizes the pre-clinical studies using pharmacological agents to target MDSCs and their proposed mechanism of action.

Targeting Strategy	Drug	Mechanism of Action	References
Prevention of MDSC formation	Curcumin derivatives	JAK/STAT3 pathway inhibition	[363–365]
	Sunitinib	Inhibition of STAT3 pathway, c-kit and VEGFR functions	[236,366–368]
	Tasquinimod	Blocks S100A9 signalling	[369]
	Vemurafenib	unknown	[370]
Induction of MDSC Differentiation	All-trans retinoic acid	Activation of the Erk pathway	[371–373]
	Vitamin D3	unknown	[374]
	Icariin derivatives	Inhibition of S100A8/9, STAT3 and Akt pathways	[375]
	MPSSS poly-saccharide	Stimulation of NFKB pathway	[376]
Blockade of MDSC Expansion	Bevacizumab	Blockade of VEGF signalling	[377]

Blockade of MDSC Activation	Anti-IFN-Y antibody	Blockade of activation	[209]
Blockade of MDSC Recruitment	GW2580	CSF1R inhibition	[265,349]
	CSF1 Neutralizing Antibody	CSF1R blockade	[350]
	COX-2/PGE-2 receptor inhibition	CXCR4/CXCL12 blockade	[352]
	Acetylsalicylic acid (ASA)	CCL2 inhibition	[351]
Blockade of MDSC Function	Zolendronic acid	c-kit cleavage	[378,379]
	Celecoxib	Inhibition of COX-2	[380]
	Sildenafil and Tadalafil	Inhibition of PDE-5	[381]
	N-hydroxy-L-Arginine (NOHA)	Inhibition of Arginase-1 function	[30]
	Nitroaspirin	Inhibition of Arginase-1 and iNOS function	[382]
	N-Acetyl cysteine (NAC)	Inhibition of ROS production	[383]
	CpG oligodeoxy-nucleotides (ODN)	Inhibition of Arginase-1 and iNOS function	[384–386]
	Bardoxolone methyl (CDDO-Me)	STAT3 inhibition	[387,388]
	Withaferin A	Stimulation of glutathione	[389,390]

Depletion of MDSCs	Monoclonal Gr-1 antibody	Targets all Gr1+ cells	[391]
	IL4Ralpha aptamer	Apoptosis via STAT6 signalling	[222,392,393]
	Gemcitabine	Induction of apoptosis and necrosis	[353,394]
	5-fluorouracil (5-FU)	Induction of apoptosis	[395,396]
	Peptibodies	Targets S100 proteins on MDSC membrane	[226]

1.5.11 Clinical Trials

Despite these promising pre-clinical studies using sunitinib, a phase III clinical trial of the TroVax vaccine for patients with metastatic renal cell cancer, which included a treatment arm containing sunitinib treatment, failed to show improvement in survival[397].

In a phase II/III clinical trial of patients with extensive small cell lung cancer, patients were randomized to undergo vaccination dendritic cells transduced with wild type p-53, no treatment, or p53 vaccine plus ATRA. ATRA was demonstrated to deplete MDSCs in treated patients and 41.7% of those treated with p53 vaccine and ATRA had IFN-Y Elispot responses to p53, compared to 20% in vaccine alone[398].

Whilst clinical trials involving MDSC depletion and immunotherapy are still in their infancy, the interest in this area is rapidly growing and there are trials recruiting in Sarcoma (DC vaccine +/-Gemcitabine), Melanoma (Ipilimumab +/- ATRA) and Head and Neck Squamous Cell Cancer (Tadafinil and Prevnar vaccine) [399].

1.6 Conclusions

While standard cytotoxic chemotherapy regimens can effectively reduce the disease burden in patients with haematologic malignancies, patients often subsequently succumb to relapsed disease due to the emergence of resistant clones [400]. In contrast, the unique potency of cellular immunotherapy in the treatment of cancer is highlighted by the observation that allogeneic transplantation is curative for a subset of patients due to the activation and expansion of alloreactive lymphocytes[401]. However targeted immune therapies have demonstrated somewhat variable clinical outcomes for patients, believed to be in part due to the suppressive tumour microenvironment. MDSCs form a critical component responsible for maintaining the immunosuppressive milieu of the tumour microenvironment. The importance of immune modulation in determining the balance between immune activation and disease has been highlighted by recent observations that PD-1 blockade alone is capable of inducing sustained disease response in patients with advanced solid malignancies[35]. Further work is needed to elucidate specific markers for MDSCs, potentially combining traditional surface and function markers with next generation modalities such as RNA and MicroRNA array derived signatures for MDSCs. Improving specificity in targeting key immune suppressive components of the tumour milieu whilst sparing tumour targeting populations, may bridge the gap between encouraging pre-clinical data, and the execution of successful clinical studies. Successfully depleting this population might hold the key to maximizing immunological responses to immune-based therapies and improving the outcomes of patients with malignant diseases.

1.7 Aims of the study

While standard cytotoxic chemotherapy regimens can effectively reduce a patient's tumour burden at the time of presentation, patients often subsequently succumb to relapsed disease, thought to be due to residual cancer cells that are inherently resistant to traditional cytotoxic therapy[400]. The unique potency of cellular immunotherapy in the treatment of cancer is highlighted by the observation that allogeneic transplantation is curative for a subset of patients due to the activation and expansion of alloreactive lymphocytes[401]. However targeted immune therapies have demonstrated somewhat variable clinical outcomes for patients, believed to be in part due to the suppressive tumour microenvironment. The importance of immune modulation in determining the balance between immune activation and disease has been highlighted by recent observations that PD-1 blockade alone is capable of inducing sustained disease response in patients with advanced solid malignancies[35]. Further work is needed to elucidate specific markers for MDSCs, potentially combining traditional surface and function markers with next generation modalities such as RNA and MicroRNA array derived signatures for MDSCs. Improving specificity in targeting key immune suppressive components of the tumour milieu whilst sparing tumour targeting populations, may bridge the gap between encouraging pre-clinical data, and the execution of successful clinical studies. Successfully depleting this population might hold the key to maximizing immunological responses to immune-based therapies and improving the outcomes of patients with malignant diseases.

To address my hypothesis that MDSCs play a role in mediating an immune suppressive microenvironment, I investigated the signalling pathways underlying the accumulation of MDSCs in AML and examine how these cells provide a tumour supporting microenvironment.

Chapter 2. Immune Suppressive MDSCs are expanded in the peripheral blood of patients with AML

2.1 Introduction

MDSCs in AML

MDSCs represent a heterogeneous population of immature myeloid cells that exert immunosuppressive effects and play a critical role in promoting immune tolerance in cancer. The critical nature of the bone marrow microenvironment in patients with AML in creating an immunosuppressive niche that fosters disease growth and immune escape has heightened interest in the role of MDSCs in AML. However, there has been very little discovered about MDSC populations or their function in patients with AML. This is likely to be due, in part, to the fact that MDSCs and AML blasts have an overlapping phenotype, making them difficult to differentiate by surface markers alone. Given that MDSCs appear to have critical importance in the tumour derived immune suppressive environment in other haematological malignancies, much effort is being made to overcome these limiting technical issues and elucidate the role of this cell in this highly lethal leukaemia.

Of note, immature myeloid cells such as MDSCs share common characteristics with myeloid leukaemia cells due to the early maturation arrest of leukaemic cells. For example, it has been suggested that AML blasts exert their suppressive effects on T cells via a similar Arginase-1 dependent mechanism to MDSCs[275]. Myelodysplastic syndrome (MDS) is a clonal disorder of the haematopoietic stem cell that increases in prevalence with age and is considered the pre-malignant condition often preceding the development of AML. It has been reported that there are increased numbers of MDSCs in the bone marrow aspirates of patients with MDS, with concurrent increases in MDSC derived TGF-β, VEGF and IL-10. The authors postulated that these expanded pool of MDSCs may have an important and pathogenic role in the development of the ineffective haematopoiesis, characteristic of MDS[216].

These collective observations lead us to investigate the presence and importance of MDSCs in AML.

2.2. Aims

The aim of this study was to quantify the MDSC population in the bone marrow and peripheral blood aspirates of patients with active AML. We further sought to elucidate if there was a requirement for MDSCs to be clonally related or distinct from the leukaemic cells. Finally, we sought to characterize the immune suppressive capabilities of these cells, in terms of their ability to suppress T cell proliferation and the polarity of T cell phenotype.

2.3. Methods

Ethical Considerations and Approvals

Ethical approval for the study was obtained from the Dana-Farber/Harvard Cancer Center Institutional Review Board. Samples of peripheral blood and bone marrow aspirates were obtained from AML patients in accordance with a protocol approved by the institutional review board. Compliance no. FWA00003245. The animal welfare assurance number was A3153-01.

Samples

Primary Samples

Peripheral blood (PB) and bone marrow (BM) aspirates were obtained from patients with Acute Myeloid Leukaemia, in accordance with a protocol as above. All patients signed informed consent forms allowing their samples to be used for research purposes. 30 millilitres (mls) of PB or 6 mls of BM aspirate was collected into EDTA tubes and used immediately. Healthy donor blood samples were obtained from the Kraft Family Blood

Donor Center (DFCI, Boston, MA). For experiments using primary AML cells, diagnostic BMs were used with a minimum of 90% blast involvement. For experiments using healthy donor PBMCs, total cell fractions were used.

Cell Lines

The human AML cells lines THP-1 and MOLM-14, and the murine AML cell line C1498 (TIB-49) were purchased from ATCC and cultured as per manufactures guidelines (see below). Cells were frozen in bulk, and fresh cells were thawed every three months for use.

The cytogenetic profile of THP-1 is as follows:
49,XY,+der(1)del(1)(p22p36)t(1;12)(p36; q11),del(6)(p21),+del(6)(p21),+8,+der(9)t(9; 11)(p22;q23)t(9;11)(q12;q23),−10,der(11)t(9;11)(p22;q23),del(12)(q11),del(17)(p11),der (20)ins(20;1)(p12;p22p36)[13]/48,idem,−del(12)(q11)[7].[402]

The cytogenetic profile of MOLM-14 is as follows:
human hyperdiploid karyotype; -49(46-50)<2n>XY, +6, +8, +13, der(2)t(1;2)(q31;q35), ins(11;9)(q23;p22p23), del(14(q23q32.3), del(16)(q11.2q13.1); sdl without der(2) but with tetrasomy 8 and/or i(8)(q10); carries insertional variant of t(9;11) recurrent in AML, effecting rearrangement of MLL (KMT2A) with MLLT3 (AF9); ins(11;9) plus trisomies 8 and 13 also present in sister cell line MOLM-13 which it closely resembles.[403]

Isolation of Peripheral Blood and Bone Marrow Mononuclear Cells by Ficoll density Centrifugation

In order to isolate PB mononuclear cells and BM mononuclear cells, samples were subjected to Ficoll density gradient centrifugation (Histopaque-1077, Sigma, St. Louis,

MO). Samples were suspended in 25mls of Roswell Park Memorial Institute (RPMI-1640) (Cellgro, Manassas, VA) and layered on top of 15mls of room temperature Histopaque in 50ml Falcon tubes. The tubes were centrifuged at 1800rpm for 25 minutes with the brake off. The interface layer representing live mononuclear cells was removed, re-suspended in 50mls of RPMI and centrifuged at 3000rpm for 10 minutes. The resulting pellet, depleted of platelets, was washed once more in RPMI and used immediately.

Cell Counting

Cell counts and viability assessment was performed using Trypan Blue (Sigma Aldrich) exclusion using a haemocytometer. 10µl of cells diluted 1:2 in trypan blue were added to the chamber of the haemocytometer. Cells were examined under a x10 objective using an Olympus CKX41 microscope. Live cells (clear) and dead cells (blue) were counted to give an estimate of viability.

Cell Freezing and Thawing

Cells were frozen by adding 1ml of Gibco Cell Culture Freezing Medium (Thermo Scientific, Waltham, MA) to pellets of $1x10^7$ cells and placing the suspension in a cryovial at -80C, or in liquid nitrogen for long-term storage. Cells were thawed rapidly at 37°C in a waterbath before the contents of the cryovial were transferred to a 50ml falcon tube. 49mls of warm RPMI were added and the tubes were then centrifuged at 1800rpm for 5minutes prior to further procedures.

Cell Culture

BM and PB mononuclear cells isolated as above and maintained in in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% bovine serum albumin or 10% exosome depleted bovine serum albumin (Sigma, St. Louis, MO) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Cellgro, Manassas, VA) and 2.5µg/mL Plasmocin

(Invivogen, San Diego, CA) and cultured at 37ºC in a humidified 5% CO_2 incubator, at concentrations of $1x10^6$ cells per ml.

Cell lines were maintained in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% human serum albumin (Sigma, St. Louis, MO) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Cellgro, Manassas, VA) and 2.5µg/mL Plasmocin (Invivogen, San Diego, CA) and cultured at 37ºC in a humidified 5% CO_2 incubator, at concentrations of $2x10^5$ cells per ml.

Flow Cytometry

Surface Staining

Samples to be stained were washed with sterile PBS, re-suspended in PBS at approximately 10^7 cells/mL and divided into FACS tubes (10^6 cells in 100µl/tube) and incubated with 1µl of each antibody, or isotype control, at 4ºC for 30 minutes. Cells were subsequently washed in PBS and re-suspended in 400µl PBS or RPMI for acquisition. Acquisition of 100,000 events was obtained using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA). A sample with no antibodies was used to set appropriate voltages. Analysis of the obtained data was performed using Kaluza software (Beckman Coulter, Brea, CA).

Gating Strategy for MDSCs

PB and BM samples were selected in which the leukaemia blast population represented a subset of the myeloid cells, allowing for the identification and selection of partially differentiated myeloid precursors distinct from the AML cells. In patients with active disease, MDSC were segregated from leukaemic cells first using Flow Cytometric analysis of size and granularity (forward and side scatter properties). In some patients the blast population is so overwhelming that it obliterates all other cell populations by flow cytometry, and it was impossible to assess these patients for MDSCs. As an example, in Figure 2, this patient has a CD14- blast population shown in gate D on the HLADR CD14 plot that obliterates the clear positive and negative populations usually seen in the CD33 CD15 plot. Compare this to the patient shown in Figure 3, whose smaller blast percentage and blast HLADR positivity renders it easier to exclude this population. MDSCs are isolated as CD33+CD15-CD11b+HLADR- (monocytic) or CD33+CD15+CD11b+HLADR- (granulocytic) and expressed as a percentage of either total cells, or as a percentage of immature myeloid cells (CD11b+, HLADR-), as per previously published studies[251].

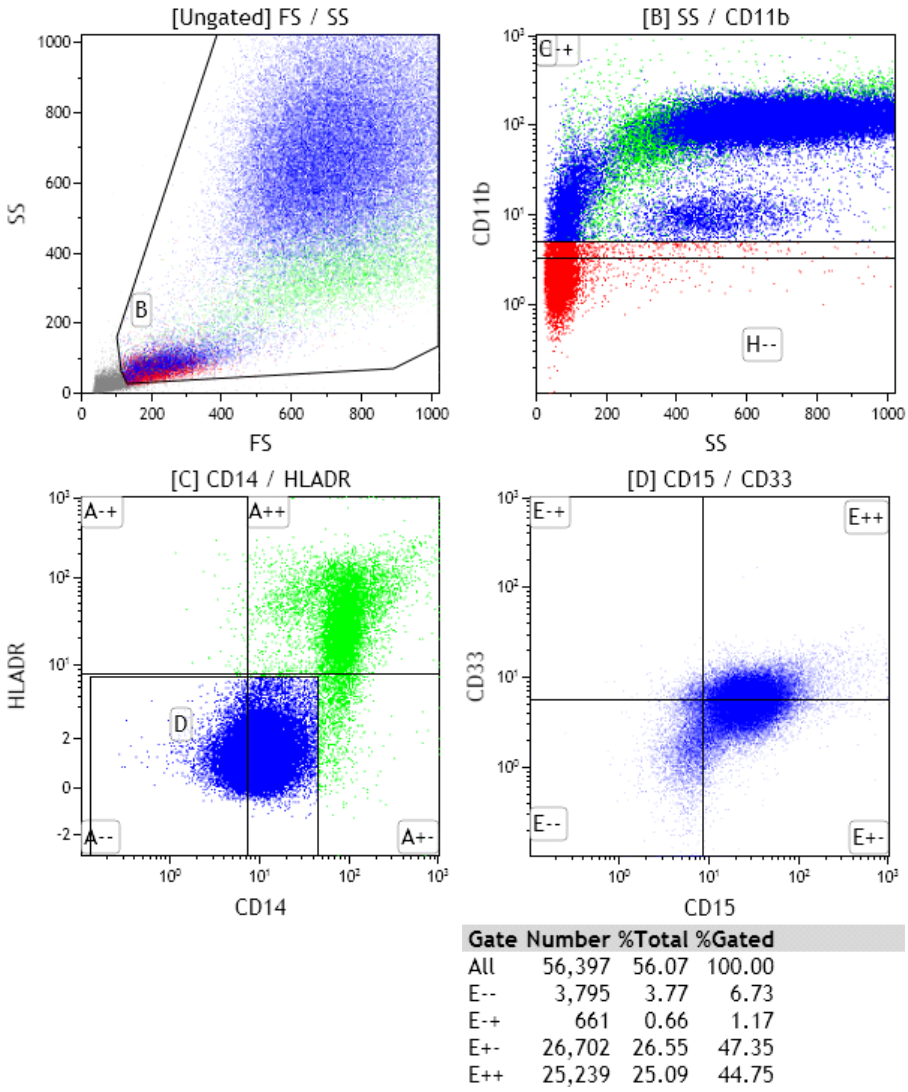


Figure 2: Flow Cytometry analysis of the peripheral blood of patient with active AML. The blast population can be seen in gate D.

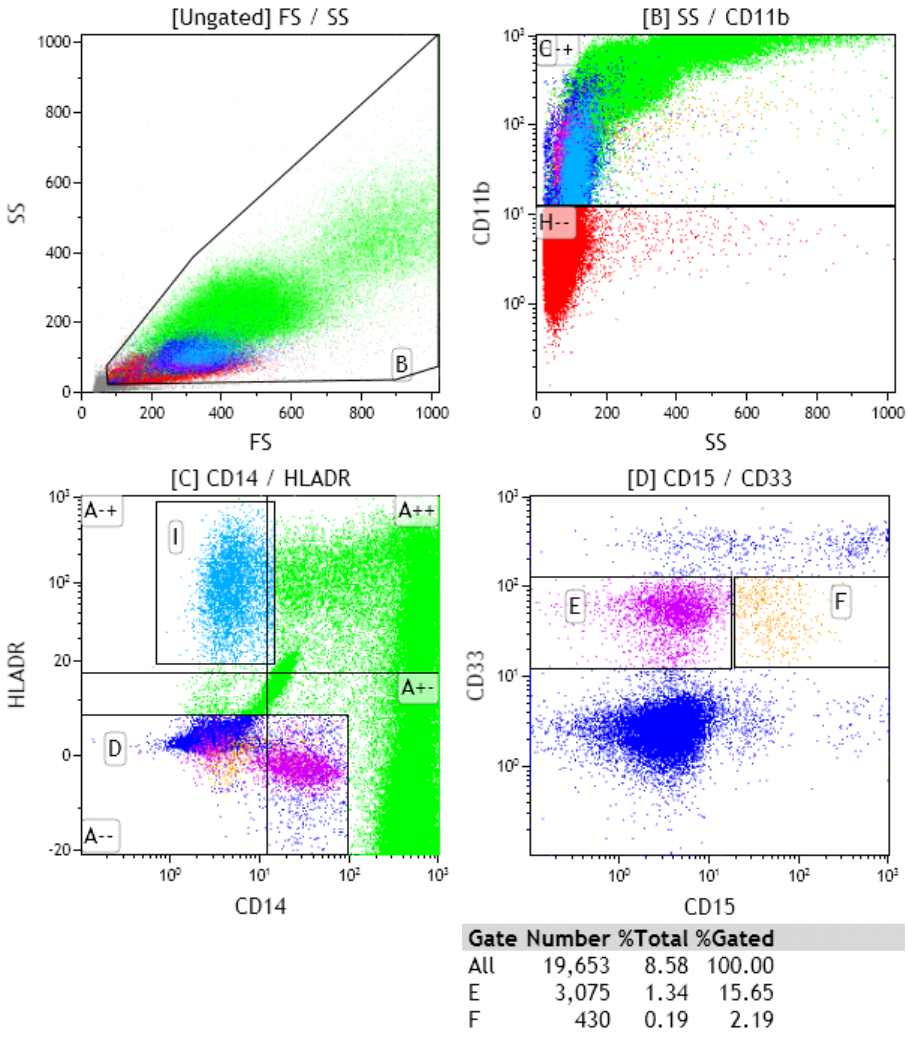


Figure 3: Flow Cytometry analysis of the peripheral blood of patient with active AML. The HLADR+ blast population can be seen in gate I. Granulocytic MDSCs are shown in gate F, and monocytic MDSCs in gate E.

Giemsa staining

MDSCs and tumour cells were isolated by FACS sorting and cytospins were prepared. Slides were fixed in methanol for two minutes and then 250ul of Wrights solution added and slides rocked for two minutes. Slides were washed in a slide bath with dH20 three

times and 250ul of Geimsa solution added for one minute. Slides were washed as previously and air dried, before being imaged.

Immune competent murine model of murine AML

$1x10^5$ GFP tagged C1498 murine AML cells were injected retro-orbitally into C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). Following establishment of disease, defined as reduced physical activity and weight loss or visible orbital tumour (chloroma) development, all mice would be euthanized. Mice started to become symptomatic at 21 days, upon which all mice were analysed. Mice were euthanized, alongside healthy control C57BL/6 mice, and their femurs and spleen were harvested. Bone marrow cells were flushed from the femur bone marrow cavity into sterile RPMI 1640 media. Spleens were emulsified to obtain a cell suspension. Bone marrow and spleen cells were washed prior to further analysis by flow cytometry. Engraftment, as defined by >1% GFP-positive cells in the BM, was detected by flow cytometry. BM and spleens were analysed by FACS for MDSC quantification using the markers CD11b and Gr1.

***In vitro* model of MDSC expansion**

PMBCs from healthy donors, isolated via Ficoll density centrifugation, were seeded in 6 well plates (Corning, NY) at $1.5x10^6$ cells per well. AML cells from cell lines or FACS isolated from primary samples from AML patients were irradiated at 7500Rad to prevent proliferation and fluorescently labelled red with GranToxiLux (OncoImmunin, MD). $1.5x10^4$ AML cells were added to test wells (ratio of 100:1). Cells were cultured at 37ºC in a humidified 5% CO_2 incubator and maintained in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% human serum albumin (Sigma, St. Louis, MO) and 100 IU/mL penicillin and 100 µg/mL streptomycin (Cellgro, Manassas, VA). After 5 days, cells were collected, rinsed twice with PBS and phenotypically analysed by flow cytometry as described, using the red dye to exclude tumour cells.

Sorting of Cells using Fluorescence Activated Cell Sorting (FACS)

To sort T cells, tumour cells and MDSCs from primary samples by FACS, samples were subjected to Ficoll density centrifugation and washed as described. Samples were labelled with markers for T cells (CD3), tumour cells (CD33, or identified via FSC and SSC), or MDSCs (CD11b, CD14, HLADR, CD33, CD15), under sterile conditions. Cell populations were identified as described and sorted using a BD FACSAria™ Fusion flow cytometer (BD, San Jose, CA).

Adherence Isolation of T cells and Dendritic Cells

To separate primary samples of peripheral blood into T cells and monocytes, PBMCs (after Ficoll density centrifugation) were re-suspended in complete media (RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% human serum albumin (Sigma, St. Louis, MO) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Cellgro, Manassas, VA)) at a concentration of $6x10^6$ cells per ml. Cells were placed in flasks and incubated for 45 minutes in a 37ºC, humidified 5% CO_2 incubator. After 45 minutes the non-adherent fraction was removed into fresh flasks and supplemented with 10 U/mL IL-2 (Roche diagnostics GmbH, Mannheim, Germany) for at least 5 days before use. The adherent fraction was cultured in complete media supplemented with 1000 U/mL GM-CSF (Berlex, Wayne/Montville, NJ, USA) and 12.5 ng/mL IL-4 (Cellgenix, Freiburg, Germany) for 5 days and 25 ng/mL TNF-α (Cellgenix, Freiburg, Germany) was added on day 5 for additional 2 days to induce maturation of dendritic cells.

Fluorescence in situ hybridization (FISH)

FISH analysis is employed to identify chromosomal aberrations in AML cells using fluorescent probes targeting a previously identified mutational sequence complementarity. We employed FISH analysis of the leukaemic clone and associated MDSC population to interrogate the clonal relationship between these cell subsets. To assess whether MDSCs may share a common derivation with pre-leukaemia malignant precursor population as compared to the leukaemia cell, we assessed mutational

patterns in MDSCs derived from two patients with MDS who evolved to AML as compared to a *de novo* case of AML.

In a PB sample obtained from patient #6 with AML evolving from MDS, the leukaemia population demonstrated the following cytogenetic abnormalities:

(45,XY,-7,del(20)(q11.2q13.3[18]/46,XY,-7,+8,del(20)(q11.2q13.3)[2]).

MDSCs and tumour cells were isolated by FACS sorting. CD15+ granulocytic MDSCs and a CD15- blast population were spun onto slides, fixed in methanol: acetic acid (3:1) and a three-colour FISH probe (Vysis LSI D20S108 SpectrumOrange labelled 201 kb DNA segment including the 20q12 D20S108 locus, Vysis D8Z2/CEP8 SpectrumGreen labelled probe specific for the alpha satellite (centromeric) region spanning 8p11.1-q11.1, and D7S486/CEP7 SpectrumAqua labelled probe specific for the alpha satellite (centromeric) region spanning 7p11.1-7q11.1) was hybridized to normal cells (NA 10851) and to fixed cytospun slides (tumour and MDSC) to assess the number of copies of chromosomes 7, 8 and 20. Chromosomal location for all three probes was confirmed using metaphases from normal sample NA 10851. Hybridization signals were clearly visualized on both cytospun samples and the normal cells.

In patient #7 with AML evolving out of MDS, the leukaemia population demonstrated the following cytogenetic abnormalities:

46,XX,del(7)(q22),del(12)(p13)[12]/46,XX[8]

A two-colour FISH probe (Cytotest D7S522/CCP7, containing the D7S522 CytoOrange-labelled DNA at 7q31.2 and CCP7 CytoGreen-labelled DNA specific for the alpha satellite (centromeric) region spanning 7p11.1-7q11.1), was hybridized to normal male cells from lymphoblastoid cell line NA10851 and to fixed cytospin slides (tumour and MDSC sorted cells) to assess presence of a deleted chromosome 7 (del(7)(q22)). Chromosomal location for the probes was confirmed using metaphases prepared from NA10851. Hybridization signals were clearly visualized in nuclei from both cytospin samples and in

nuclei from the normal sample.

Immunohistochemistry

MDSCs and tumour cells were isolated from patient #8 by FACS sorting. CD15+ granulocytic MDSCs and a CD15- blast population were spun onto Poly-L-Lysine coated slides (LabScientific, Inc., NJ) and fixed in 4% PFA for 7 minutes and stored in PBS at 4°C. Slides were first treated with 2cc /slide of H_2O_2 for 15 minutes to block endogenous peroxidase activity. Slides were washed with PBS and incubated at room temperature in a humidified case, with 200µl primary antibody against NPM1 (Dako, CA) for 30 minutes. Slides were washed in PBS and incubated for 30 mins at room temperature with 200µl HRP labelled polymer anti-mouse secondary antibody (DAKO Envision + System HRP, Dako CA). Slides were washed in PBS and developed in Benzidine before being imaged.

Proliferation Assays

MDSCs were FACS isolated, from patient samples as described. T cells autologous to the MDSCs were isolated by non-adherence, as described. Stimulation of T cells with anti-CD3/CD28 was accomplished by coating 24-well non-tissue culture-treated plates (Falcon, Fisher, Pittsburgh, PA, USA) or 96-well opaque flat bottom plates (Corning, NY) with anti-CD3 (clone-UCHT1; Pharmingen, San Diego, CA, USA) and anti-CD28 (clone-CD28.2; Pharmingen, San Diego, CA, USA) at the concentration of 1 µg/mL in phosphate-buffered saline (PBS) at 0.3 mL/well overnight at 4 °C. The plates were washed in 1 × PBS, and T cells and autologous MDSCs were co-cultured at a ratio of 2:1 (Tc:MDSC), $4x10^5$-$1x10^6$ T cells per well, in 200µl of complete media. After 3 days in culture at 37ºC in a humidified 5% CO_2 incubator, the analysis was as follows. Cells 24-well plates were analysed for the expression of markers of T cell activation (CD69-PE, CD25-FITC, CD4-PB) by flow cytometry as described.

Intracellular Staining

The expression of inflammatory (IFN-γ) and inhibitory (IL-10) cytokines was analysed by intracellular flow cytometry. T cells were pulsed with GolgiStop (1 µg/mL; Pharmingen,

San Diego, CA, USA) for 4–6 h at 37 °C prior to analysis. Cells were next harvested and washed with RPMI and re-suspended in PBS at approximately 10^7 cells/mL and divided into FACS tubes (10^6 cells in 100µl/tube) and incubated with 1µl of CD4-PB and CD8-FITC at 4ºC for 30 minutes. Cells were washed with PBS and then then permeabilised by incubation in 200µl Cyto-fix/Cytoperm plus™(Pharmingen, San Diego, CA, USA) containing formaldehyde and saponin for 30 min at 4 °C, before being washed in Perm/Wash™ solution (Pharmingen, San Diego, CA, USA). Subsequently cells were incubated with 2 µl PE-conjugated IFN-γ (Invitrogen, Camarillo, CA, USA), IL-10 (eBioscience, San Diego, CA, USA), or a matched isotype control antibody, in 100 µl Perm/Wash™, for 30 min at 4ºC. Cells were washed in 1 × Perm/Wash™ solution and re-suspended in either 400 µl Perm/Wash™ (if analysed immediately) or fixed in 400 µl 2% paraformaldehyde (Sigma, St. Louis, MO, USA) (if to be stored before analysis).

CellTiterGlo Proliferation Assay

Cells in 96 well plates were analysed for proliferation by bringing plates to 21°C and adding 50µl of CellTiterGlo Cell luminescence Assay (Promega, WI) and agitating on a rocker platform for 2 minutes. Plates were read using an Infinite M200 Pro luminometer (Tecan, Mennedorf, Switzerland) and the values converted into a proliferation index (P.I) by dividing all experimental well values by the control well values.

Statistical Analysis

The Student's t-test was used to assess statistical significance.

2.4. Results

MDSCs are expanded in patients with AML

PB from AML patients was analysed for the presence of MDSCs by flow cytometry and compared to healthy controls. Ten PB samples from AML patients were discarded as unsuitable due to the blast count obliterating all other cell populations as described

above (representative plots shown in Figure 2). Patient characteristics are summarized in Table 4. Total MDSCs were defined as CD11b+ CD33+ $HLADR^{low/-}$ with the gating strategy show in Figure 4A (patient #6). As some groups use Lineage negativity to further define MDSCs as CD11b+ CD33+ $HLADR^{low/-}$ Lin^{-}, we additionally validated that in AML, CD11b+ CD33+ $HLADR^{low/-}$ MDSCs were predominantly Lin^{-}, with a mean of 89% of CD11b+ CD33+ $HLADR^{low/-}$ MDSCs being Lin^{-} (n=3), representative blot shown (Figure 4A). As such, in subsequent experiments we did not use a Lineage antibody in the antibody cocktail.

When expressed as a percentage of total cells, patients with active AML (patients #1-8) had 7.94% MDSCs (range 1.70-17.0) compared to 0.2% in healthy patients (range 0.02-0.88), $p<0.05$ (Figure 4B). When expressed as a percentage of immature myeloid cells (CD11b+, $HLADR^{low/-}$), as in all subsequent graphs, patients with AML had a mean of 23% MDSCs (range 6-40) compared to 5.2% in healthy patients (range 0-15), $p<0.05$. Upon further characterization, these MDSCs were predominantly CD15+ Granulocytic MDSCs, with patients with active AML having a mean Granulocytic MDSC percentage of 17.2% (range 2-42) compared to 1.8% in healthy patients (range 0-7.8), $p=0.01$ (Figure 4C). Monocytic subsets of MDSCs showed a trend toward expansion in patients with AML. Of note, patients with active AML had Granulocytic MDSCs present in bone marrow aspirates 24.2% (range 0-82) (n=9) (Figure 4D).

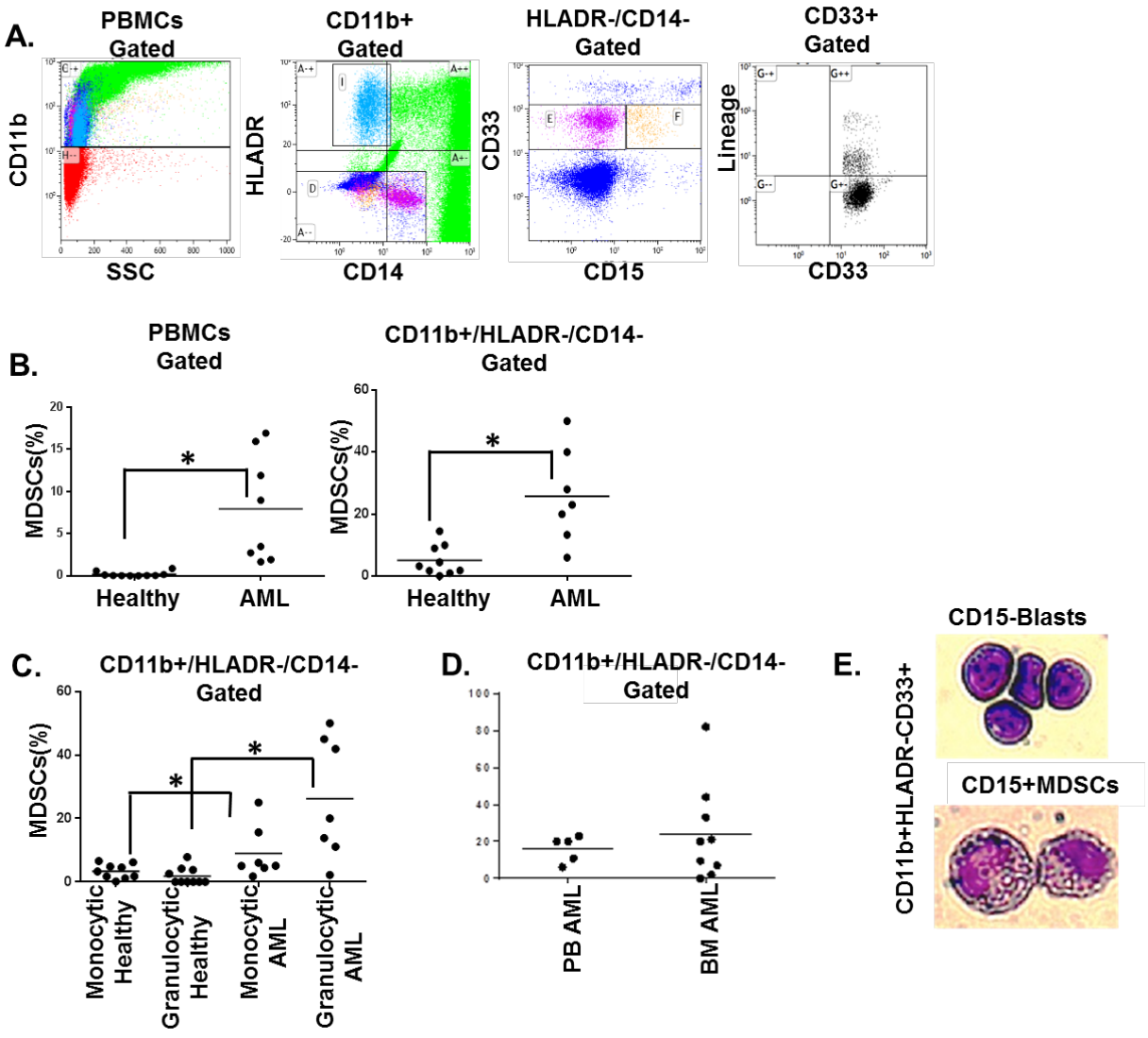


Figure 4. MDSCs are expanded in patients with AML. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and stained with antibodies for CD11b, HLADR, CD14, CD15 and CD33 expression. The cells were then analysed by Flow Cytometry. **(A)** Representative example of patient #2 is shown. CD11b+ HLADR+ CD14- blasts are shown in gate 'I' (light blue). Monocytic MDSCs (CD11b+ HLADR- CD14-/+ CD33+ CD15-) are shown in gate 'E' (purple), Granulocytic MDSCs (CD11b+ HLADR- CD14-CD33+CD15+) are shown in gate 'F' (orange). **(B)** PBMCs

from AML patients and healthy controls were isolated by Ficoll density gradient centrifugation and stained with antibodies for CD11b, HLADR, CD14, CD15 and CD33 expression. The cells were then analysed by Flow Cytometry. If present, tumour cells were gated out based on forward scatter/side scatter and known blast phenotype, and total MDSCs (CD33+CD15- or CD33+CD15+) were quantified as a percentage of total cells (n=8; $p<0.05$), and as a percentage of gated immature CD11b+/HLADR- myeloid cells (n=7; $p<0.05$). **(C)** MDSCs were further characterized as Granulocytic, by the presence of CD15+, or monocytic, by CD15- and side scatter. Granulocytic and Monocytic MDSCs in AML (n=7) vs. healthy donors (n=9) is shown. MDSCs are shown as a percentage of gated immature CD11b+/HLADR- myeloid cells. $p<0.05$ for both monocytic and granulocytic MDSCs in AML vs. healthy donors. **(D)** MDSCs were quantified, as described, in the bone marrow aspirates from patients with AML, and compared to peripheral blood. **(E)** After sorting, MDSCs were fixed on cytospin slides and stained with standard Wright-Giemsa stain to allow for morphologic evaluation, and compared to primary leukaemia cells. Representative cells are shown.

Table 4

Patient Number	% Bone Marrow Blasts	WCC at diagnosis	Diagnosis/ Relapse	FLT3/ITD	NPM	Karyotype	AML Phenotype
1	30	5.3	relapsed	-	-	normal	CD11b- HLADR- CD33+
2	84	15.5	diagnosis	+	-	normal	CD11b- HLADR+ CD33+/-
3	94	102.3	diagnosis	-	-	normal	CD11b- HLADR- CD33+

4	93	37.4	diagnosis	-	-	Complex	CD11b+ HLADR+ CD33+
5	40	8.9	relapsed	+	-	Complex	CD11b+ HLADR+ CD33+
6	42	60.6	diagnosis	-	n/a	Complex **(-7,+8,-20)**	CD11b+ HLADR+ CD33+/-
7	69	4.3	diagnosis	-	+	Complex **(-7)**	CD11b- not available HLADR+ CD33+
8	45	12.3	diagnosis	-	+	normal	CD11b+ HLADR+ CD33+

MDSCs can be expanded from mononuclear cells by AML blasts

Given these findings we sought to further characterize the nature of MDSC expansion in AML. In a murine model, leukaemic engraftment was associated with increased presence of MDSCs in the spleen and BM. Mice underwent retro-orbital injection with $1x10^5$ cells of the syngeneic murine AML cell line, TIB-49. After three weeks, at the onset of symptomatic disease, mice were euthanized and assessed for engraftment (as defined by >1% GFP+ cells in the bone marrow) and the presence of murine MDSCs. All TIB-49 injected mice engrafted leukaemia with a mean of 8% GFP+ AML cells in BM and 2.5% in spleen ($p<0.05$). Mice engrafted with AML demonstrated a significant expansion in bone marrow ($p=0.02$) and splenic MDSCs ($p<0.05$), compared to healthy mice (Figure 5A).

We next sought to characterize the effect of human AML cells on MDSC expansion *in vitro*. Co-culture of healthy donor PBMCs with AML cells for 5 days resulted in significant fold expansion of cells exhibiting an MDSC phenotype. Similar findings were observed using human AML cell lines and primary patient derived AML cells, in which patients #2-4 were used (Figure 5B). Of note, AML mediated expansion of MDSCs was abrogated in

a Transwell system consistent with the requirement for direct cell contact as opposed to an effect mediated by soluble factors derived from the tumour (Figure 5C). An expansion of MDSCs was also seen in an autologous system, whereby patient #7 blasts were cultured with patient #7 PBMCs (once remission was achieved) (Figure 5D), Absolute frequencies of cells are shown in Appendix F.

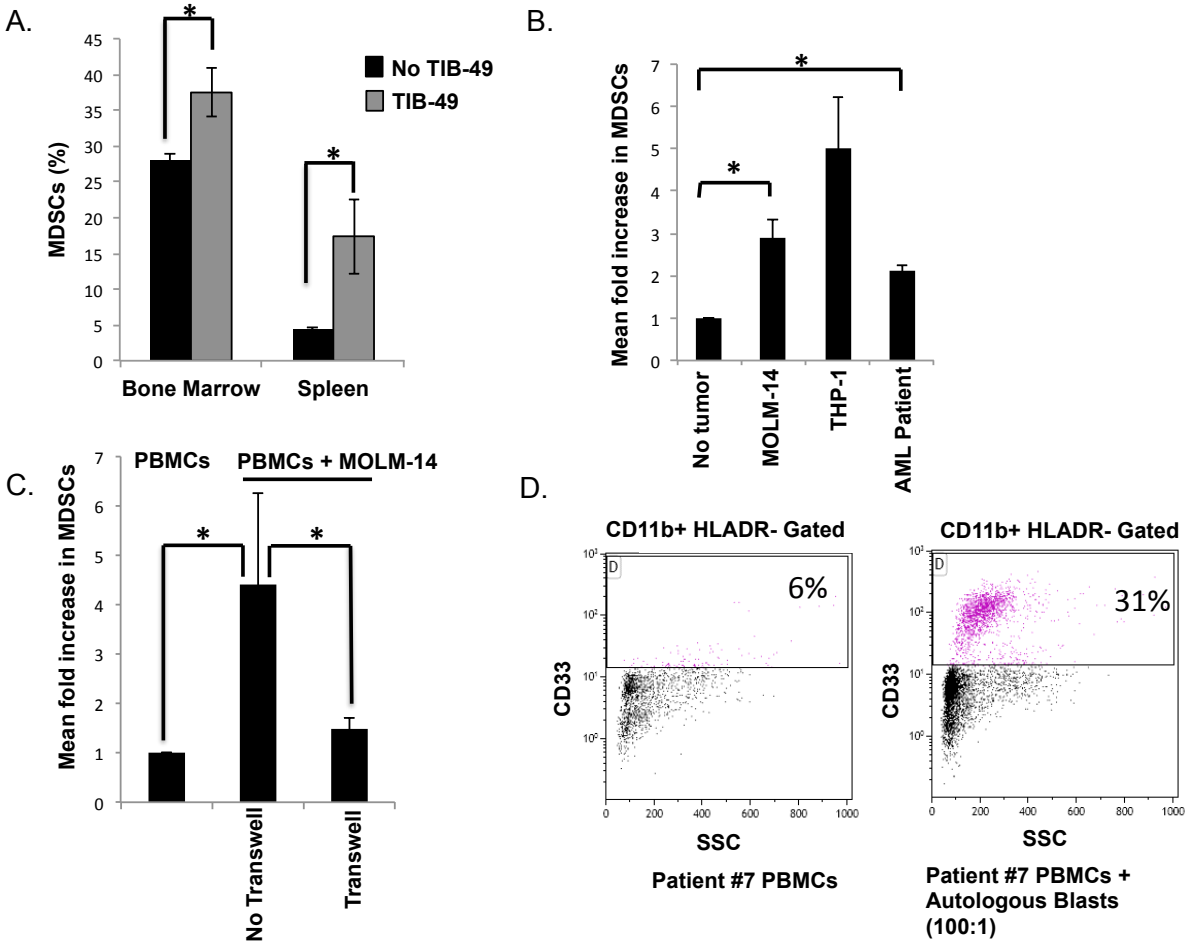


Fig 5. MDSCs are expanded in the presence of AML blasts. C57BL/6 mice were inoculated using retro-orbital injections, with $1x10^5$ GFP stably transduced murine syngeneic AML TIB-49 cells. At the onset of symptomatic disease at 21 days, mice were analysed. **(A)** Bone marrow and splenocytes were analysed by flow cytometry for the murine MDSC markers mCD11b and mGr-1 (n=5, $p<0.05$). **(B)** PMBCs from healthy

donors were co-cultured in direct contact with irradiated, fluorescently labelled AML cells at a ratio of 100:1. After 5 days, cells were analysed by flow cytometry. Labelled tumour cells were excluded and total MDSCs were quantified as a percentage of immature CD11b+/HLADR- myeloid cells (n=3, $p<0.05$ for MOLM-14 and patient AML cells). **(C)** Healthy donor PBMCs and AML cells were co-cultured in direct contact or with Transwell insert and MDSCs were quantified as a percentage of immature CD11b+/HLADR- myeloid cells (n=3, $p<0.05$). **(D)** PMBCs from (patient #7) an AML patient in remission, were co-cultured in direct contact with irradiated, autologous AML cells at a ratio of 100:1. After 5 days, cells were analysed by flow cytometry. HLADR+ tumour cells were excluded and total MDSCs were quantified as a percentage of immature CD11b+/HLADR- myeloid cells.

In a patient with MDS-AML, MDSCs were clonally related to the leukaemic blast population

FISH analysis was performed on FACS sorted AML blasts and MDSCs from the peripheral blood of patient #6, who had MDS-AML.

A total of 100 nuclei were scored for tumour, MDSCs and normal cells, 50 cells by each of two observers. However, due to a low cell count found on the MDSCs, only 88 nuclei could be scored, 43 and 45 cells by first and second observer respectively. A normal signal pattern of 2-Orange, Green and Aqua signals was observed in 86% (86/100) of cells scored in sample NA 10851 (normal sample Figure 6A). Our FISH results on the tumour (Figure 6B) and MDSC cells (Figure 6C) confirmed monosomy of chromosome 7, with a single Aqua signal noted in 98% (98/100) of the tumour and 98.8% (87/88) of the MDSC cells. Three green signals were observed in 53% (53/100) of tumour cells, consistent with trisomy for chromosome 8. A normal signal pattern for chromosome 8 (2-Green signals) was noted in 98.8% (87/88) of the MDSC cells. A deletion of the 20q12 region was also confirmed as a single Orange signal pattern was noted in 98% (98/100) and 81.8% (72/88) of tumour and MDSC cells, respectively. Notably, the MDSC sample

also had ~14.8% (13/88) cells with no Orange signal, indicative of a deletion that had occurred in both copies of chromosome 20 (q12 region), or potentially loss of one or both copies of chromosome 20.

In summary, the AML blasts contain 3 cytogenetic abnormalities, del7 – strongly associated with the MDS phenotype, del 20, and trisomy 8. The "MDSCs" however, only have del 7 and del20. These cytogenetic results suggest that in this particular patient with MDS, the MDSCs may have preceded the development of the AML blast population, and are related to the underlying MDS leukaemia precursor clone.

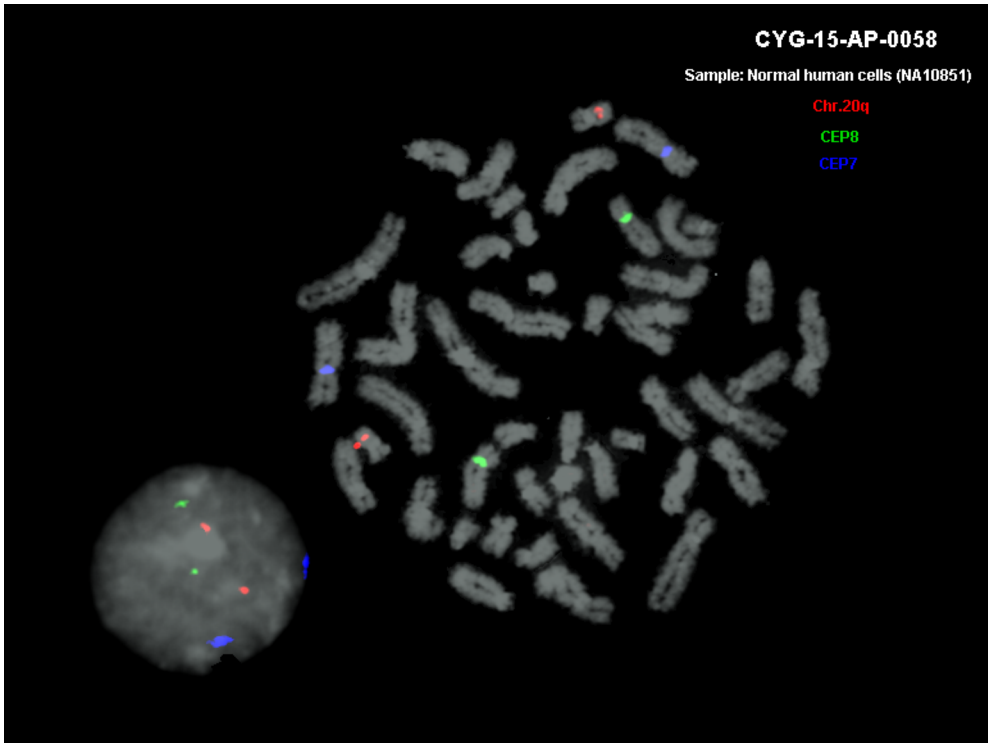


Fig 6A. Healthy control cells

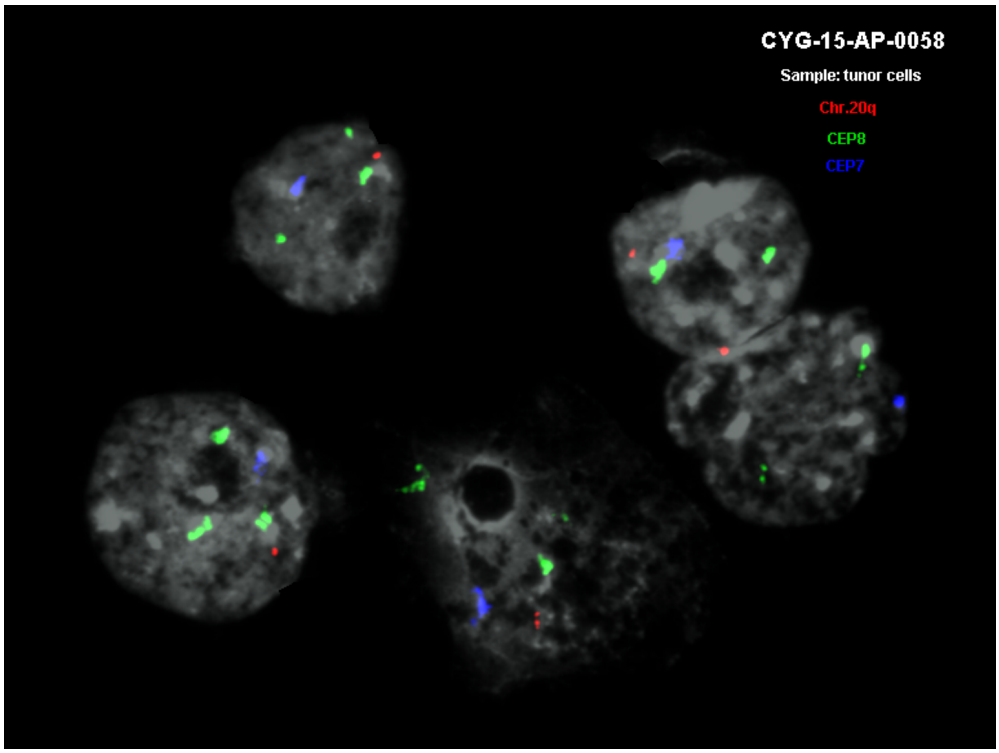


Fig 6B. AML blasts

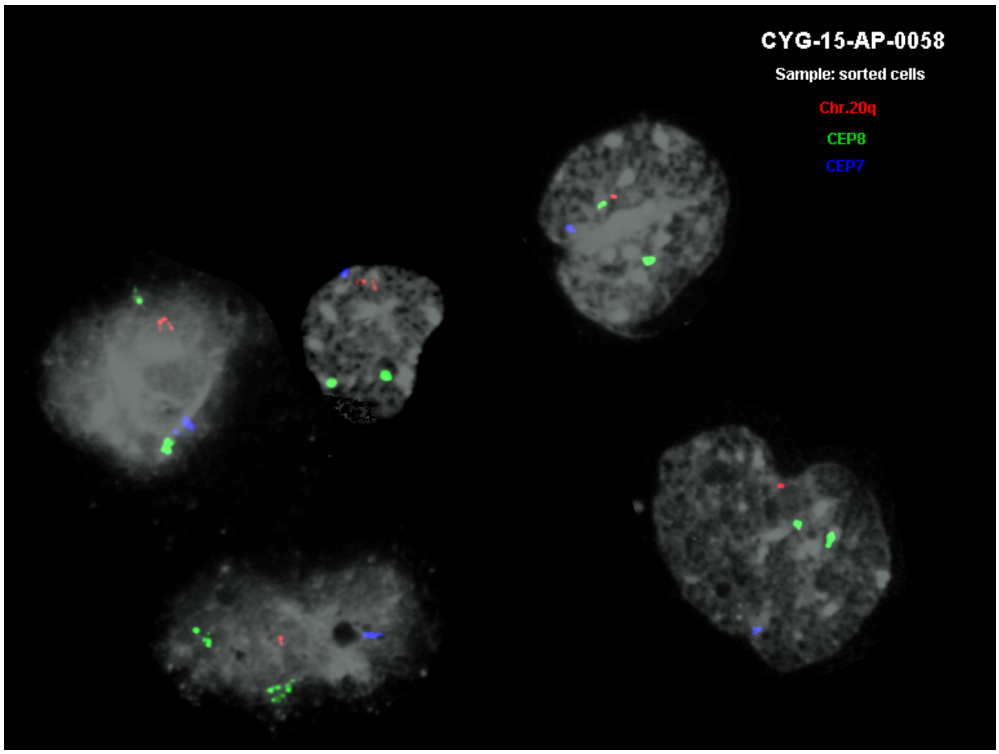


Fig 6C. Granulocytic MDSCs

Sample	Signal pattern	Number of nuclei =%
Patient #6 Tumour	1Or 3Gr 1Blu	50
	1Or 2Gr 1Blu	45
	2Or 3Gr 1Blu	1
	1Or 3Gr 2Blu	1
	2Or 1Gr 1Blu	1
	1Or 3Gr 2Blu	1
	1Or 4Gr 1Blu	1
Total		100

Sample	Signal pattern	Number of nuclei
Patient #6 MDSC	1Or 2Gr 1Blu	70
	0Or 2Gr 1Blu	13
	2Or 2Gr 1Blu	3
	1Or 3Gr 1Blu	1
	1Or 2Gr 2Blu	1
Total		88

Fig 6D. Frequencies of Cells

Figure 6. MDSCs were cytogenetically related to the AML blasts, in a patient with MDS-AML. MDSCs and blast cells were isolated using Flow Cytometry sorting from peripheral blood sample from a patient with active AML. FISH analysis was performed for known cytogenetic abnormalities. The cells were probed for del7 (blue), trisomy 8 (green) and del20 (red). 100 cells were scored and healthy control cells **(A)**, AML blasts **(B)** and MDSCs **(C)** are shown as representative examples. **(D)** Frequencies of Cells shown are tabulated.

In patient #7 with MDS-AML, MDSCs were clonally related to the leukaemic blast population

FISH analysis was performed on FACS sorted AML blasts and MDSCs from the peripheral blood of a patient with MDS-AML.

A total of 100 nuclei were scored for each sample, 50 cells by each of two observers. A normal signal pattern of two Orange (Or) and two Green (Gr) signals was observed in 96% (96/100) of nuclei scored from the normal sample; a single “false positive” cell (1/100) with the 1Or 2Gr signal pattern expected from a del(7)(q22) was seen (Figure 7A).

FISH results on the tumour (Figure 7B) and MDSC sorted cells (Figure 7C) confirmed presence of the del(7), with the 1 Or 2 Gr signal pattern noted in 92% (92/100) of the tumour cells. In the MDSC sorted specimen, approximately equal numbers of the normal 2 Or 2 Gr pattern and the del(7) 1 Or 2 Gr signal pattern were observed in these cells.

In summary, the AML blasts contain del7 – strongly associated with the MDS phenotype. The “MDSCs” however, were comprised of 50% of cells with normal chromosome 7 and 50% del 7. These cytogenetic results suggest that in this particular patient with MDS, the MDSCs may be clonally normal or abnormal.

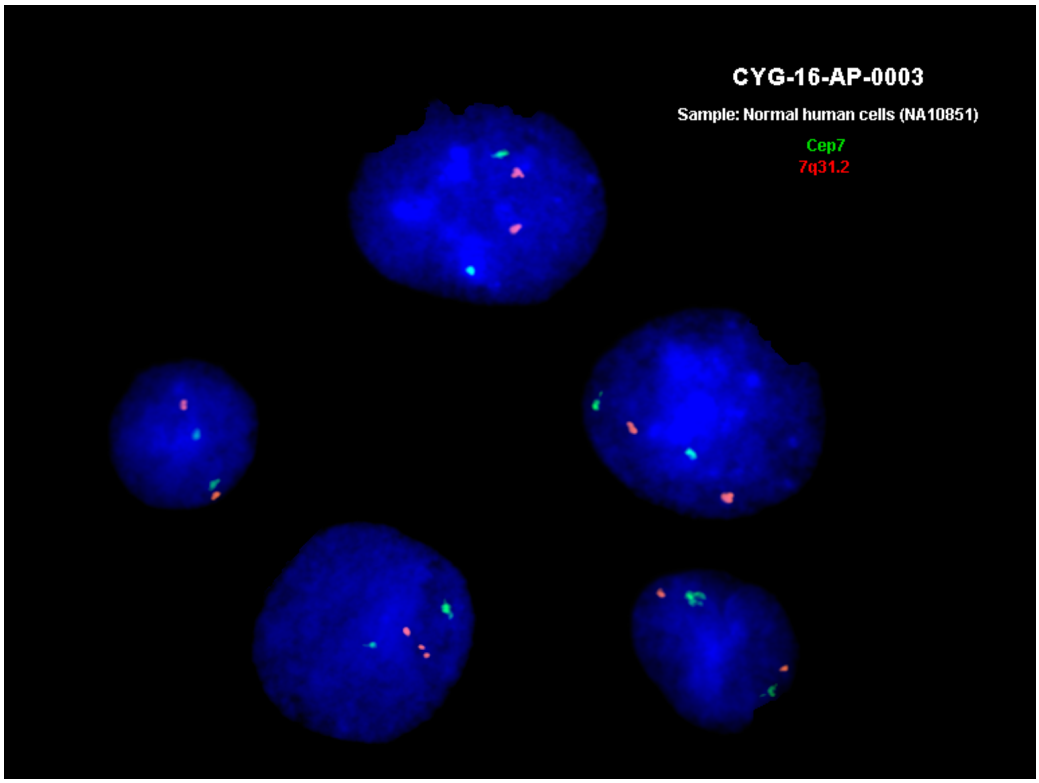


Fig 7A. Healthy control cells

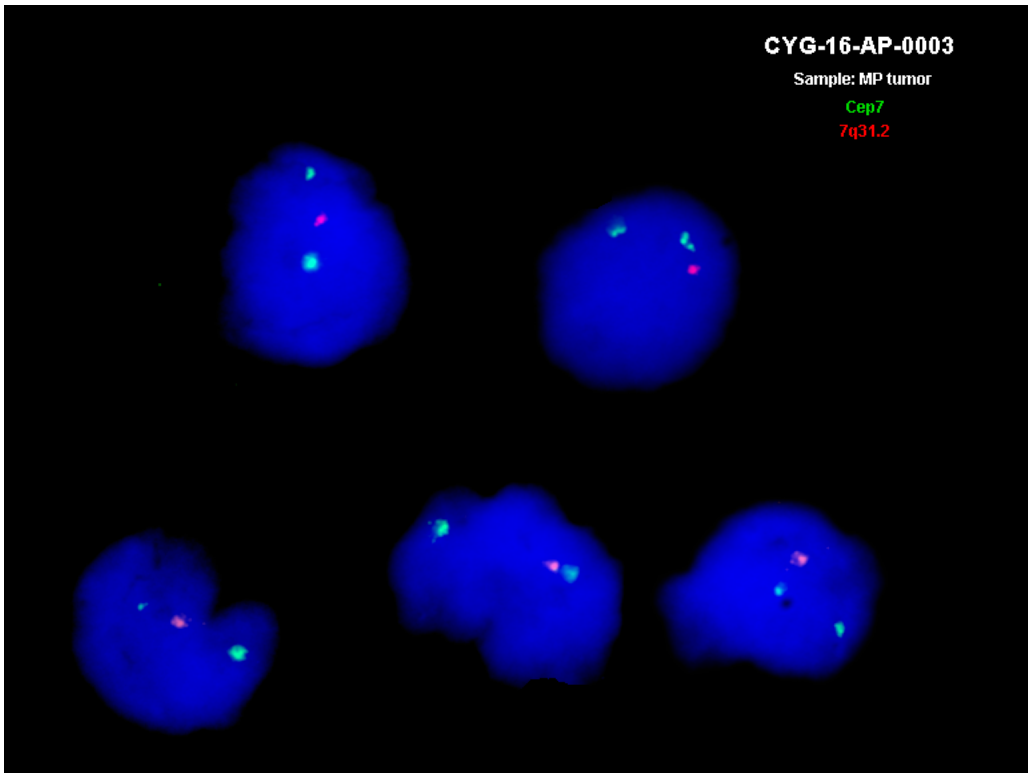


Fig 7B. AML blasts

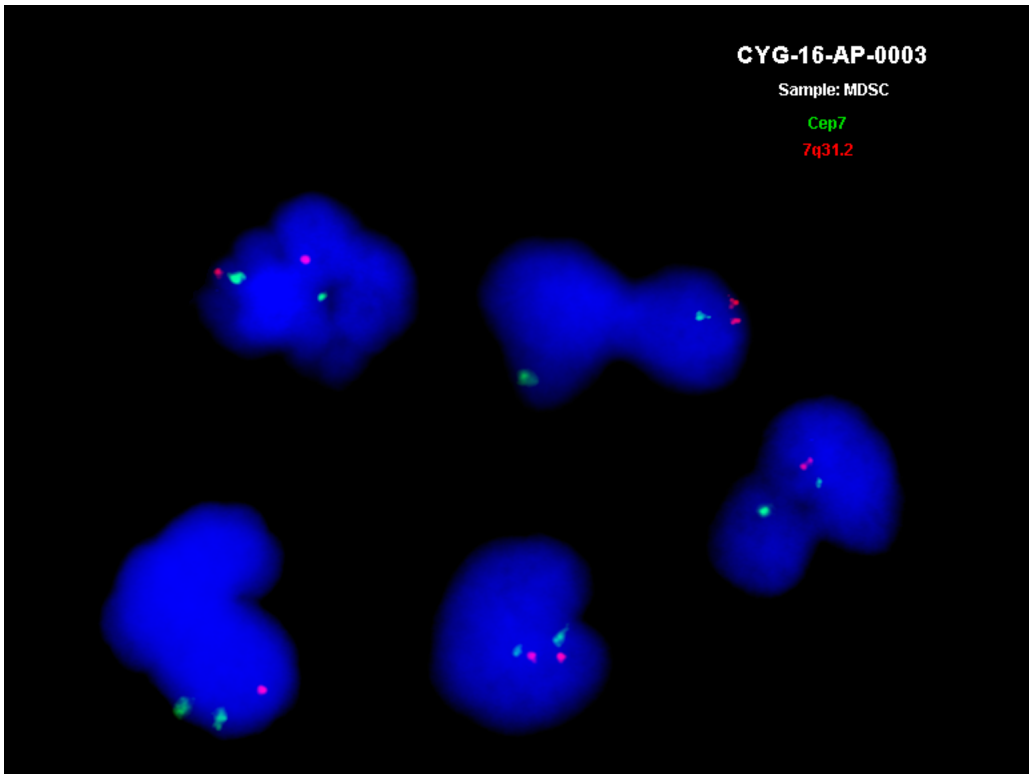


Fig 7C. Granulocytic MDSCs

Sample	Signal pattern	Number of nuclei =%
Patient #7 tumour	2Or 2Gr	6
	1Or 2Gr	92
	1Or 1Gr	2
Total		100

Sample	Signal pattern	Number of nuclei =%

Patient #7 MDSC	**2Or 2Gr**	**45**
	1Or 2Gr	**50**
	2Or 1Gr	**4**
	1Or 1Gr	**1**
Total		**100**

Fig 7D. Frequencies of Cells

Figure 7. MDSCs were cytogenetically related to the AML blasts, in a patient with MDS-AML. MDSCs and blast cells were isolated using Flow Cytometry sorting from peripheral blood sample from a patient with active AML. FISH analysis was performed for known cytogenetic abnormalities. The cells were probed for del7 (red). 100 cells were scored and healthy control cells **(A)**, AML blasts **(B)** and MDSCs **(C)** are shown as representative examples. **(D)** Frequencies of Cells shown are tabulated.

In a patient with NPM1 mutated, cytogenetically normal AML, MDSCs were clonally distinct from the leukaemic blast population

Similarly, we explored the derivation of MDSCs in a patient de-novo leukaemia characterized by the presence of a NPM1 mutation and associated more differentiated phenotype. MDSCs were FACS sorted from a peripheral blood sample from a patient (patient #8) with active NPM1 mutated AML and immuno-histochemically stained for NPM1. Wildtype NPM1 localizes only to the nucleus whereas mutated NPM1 is visible throughout the cytoplasm. The NPM1 mutated AML cell line OCI/2 was used as a positive control for NPM1 mutant (Figure 8A), and the NPM1 wildtype AML cell line THP-1 was used as a control for NPM1 WT (Figure 8B). The patients blasts stained

positive for mutant NPM1 (Figure 8C) and the sorted MDSCs stained for wildtype NPM1 (Figure 8D). As a very small number of MDSCs were sorted from this patient sample (13,000 cells) the cytospin was sparsely populated with cells and a representative sample of three cells in shown in Figure 8D. Although this experiment was limited by the small number of assessable MDSCs, these results suggest that in this patient, the MDSCs may be clonally distinct from the AML blasts.

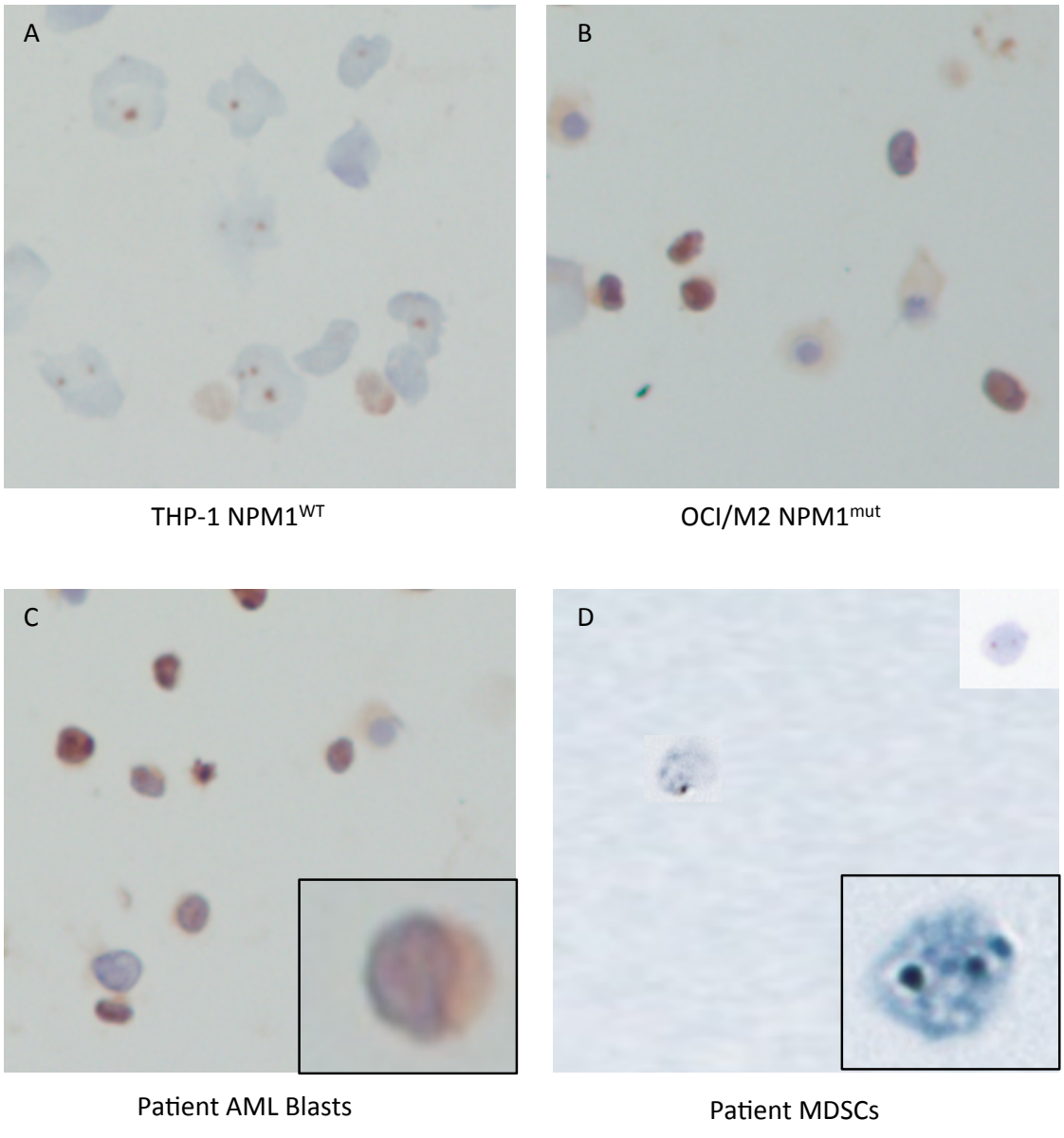


Figure 8. MDSCs were cytogenetically distinct to the AML blasts, in a patient with de novo NPM1^{mut} AML. AML blasts and MDSCs were FACS sorted from a patient with

$NPM1^{mut}$ AML and stained for mutant NPM1 (red) using IHC. NPM1 mutated AML cell line OCI/2 is shown as a positive control for NPM1 mutant **(A),** and the NPM1 wildtype AML cell line THP-1 was used as a control for NPM1 WT **(B).** 20 cells were scored in patients blasts **(C)** and sorted MDSCs **(D).**

MDSCs are suppressive of T cell activation and proliferation.

We subsequently interrogated the functional properties of the AML induced MDSC population with respect to their immunosuppressive characteristics. T cells were stimulated with CD3/CD28 and co-cultured with autologous MDSCs. After three days in culture, cells were analysed for proliferation. T cells in co-culture with THP-1 and MOLM-14 derived MDSCs were suppressive of T cell proliferation, showing a 45% and 70% reduction in the proliferative index, respectively (Figure 9A).

T cells in co-culture with autologous MDSCs showed a 46% reduction intracellular expression of CD8 IFN-γ (Figure 9B), a 40% reduction in the expression of the markers of T cell activation (CD69, CD25, CD4) (Figure 9C), and a 12 fold concurrent increase in CD4 intracellular expression of the inhibitory cytokine IL-10, as shown by flow cytometry (Figure 9D). Absolute frequencies of cells are shown in Appendix F.

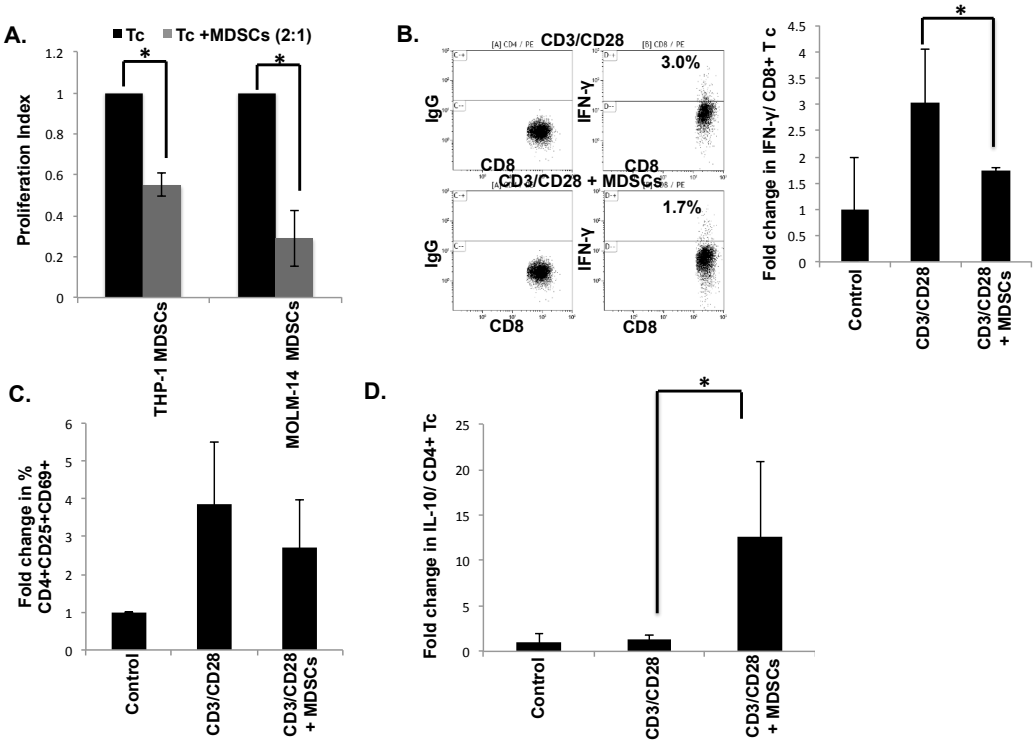


Fig 9. MDSCs are suppressive of T cell activation and proliferation. Healthy PBMCs and AML cells were co-cultured for five days and MDSCs were isolated by Flow Cytometric sorting. T cells autologous to the MDSCs were isolated and stimulated with anti-CD3/CD28 ligation. MDSCs were added at a 2:1 ratio (Tc:MDSCs). **(A)** After three days of culture, T cells were analysed for proliferation using CellTiterGlo Cell luminescence assay (n=3, $p<0.05$). After 3 days of culture cells were analysed for **(B)** the expression of intracellular IFN-γ by flow cytometry as presented in a representative experiment (left) and summary of three independent experiments (right, $p<0.05$) and **(C)** markers of T cell activation CD4+/CD25+/CD69+ (n=3) and **(D)** the expression of intracellular IL-10 by flow cytometry, as presented as a summary of three independent experiments ($p<0.05$).

2.5. Discussion

These data collectively demonstrate that MDSCs are increased in the PB of patients with AML as compared to normal individuals, and are present in their BM. It should be noted that the average age of AML patients was 68, and the healthy donor samples would likely be from younger people who donated blood at the blood centre. There is some of data to suggest that absolute numbers of MDSCs increase with age[404], and our findings should therefore be interpreted in this context.

Direct contact co-culture of tumour cells and healthy donor PBMCs elicited a robust expansion of MDSCs suggesting that tumour cells induce MDSCs, perhaps to protect their immune privileged niche. This finding was also observed in immune-competent mice engrafted with leukaemia. These observations are in keeping with similar studies in solid tumours, where co-culture of tumour cells with donor PBMCs elicited similar repsonses[256]. These MDSCs, so far defined by surface markers, were functionally suppressive of autologous T cell proliferation and promoted a switch from a Th1 to a Th2 T cell phenotype, fulfilling the “Suppressor” part of their name.

We next sought to determine if MDSCs in AML were clonally related to the leukaemic cells or were derived from normal myeloid precursors in which the tumour microenvironment favoured their selective expansion. MDSCs isolated by flow cytometric sorting were interrogated for the presence of cytogenetic or molecular abnormalities that had previously been identified in the leukaemic clone. These studies demonstrated diversity with respect to the origin of the MDSC population suggesting they did not exclusively arise from the leukaemic clone. In a patient with AML cells exhibiting 3 cytogenetic abnormalities, (del7, del20, and trisomy 8), MDSCs expressed only del 7 and del20 suggesting a common clonal origin with leukaemic precursor prior to the attainment of the trisomy 8 mutation. In a second patient with del7 AML, only 50% of the MDSCs exhibited the cytogenetic abnormality consistent with mix derivation from malignant and non-malignant myeloid precursors. In a third patient with AML cells

characterized by a NPM1 mutation, the MDSC population was found to have the wild type form of NPM1 suggestive of a lack of common clonal derivation with the AML population. While deep sequencing of these apparently clonally distinct or "normal" myeloid cells are likely to uncover the early genomic abnormalities pervasive in the haematopoietic landscape of AML[405], these studies suggest that MDSC expansion in the AML microenvironment may not be solely a reflection of myeloid differentiation of the leukaemic clone but may also result from an effect on myeloid cells in the bone marrow niche irrespective of their derivation.

It remained unclear as to how AML cells elicit the expansion of MDSCs *in vivo* and *in vitro*. Previous investigators in other cancer models have shown the importance of soluble tumour derived factors such as GMCSF and IL-1 and IL-6[256], however we observed that MDSC expansion was abrogated in Transwell plates suggesting that in AML, MDSC expansion is not purely cytokine mediated. Transwell plates prevent direct cell-cell contact between cells in each chamber, and also, at 0.4um pore diameter, impair the passage of extra-cellular vesicles[406,407]. Tumour secreted extra-cellular vesicles have been demonstrated to be an important mediator of MDSC expansion[272,334]. In the next chapter we detail our efforts to elucidate the role of tumour derived extra-cellular vesicles in modulating the immune microenvironment in AML.

Chapter 3. AML cell secrete extra-cellular vesicles that skew surrounding cells towards an immune suppressive phenotype

3.1 Introduction

Extracellular vesicles (EVs) are membrane bound vesicles released by virtually all cells and which can be found in the majority of bodily fluids. EVs have a complex nomenclature, whereby they can be defined by size, with *exosomes* defined as 40-100nM, and *microvesicles*, defined as 100-1000nM[269–271]. Furthermore they can also be defined by function or cell of origin, with the term *oncosome* used to describe tumour derived vesicles[408].

3.1.1 Biogenesis of EVS

Some vesicles originate by straightforward budding of the cell's external plasma membrane, while others originate by "reverse budding" from the late endosomal compartment, which then becomes known as a multivesicular body (MVB) or a multivesicular endosome (MVE). As shown in Figure 10, the MVE then moves to the periphery of the cytosol, where it fuses with the cell membrane, allowing the spilling of the vesicles contained within it[269,407,409,410].

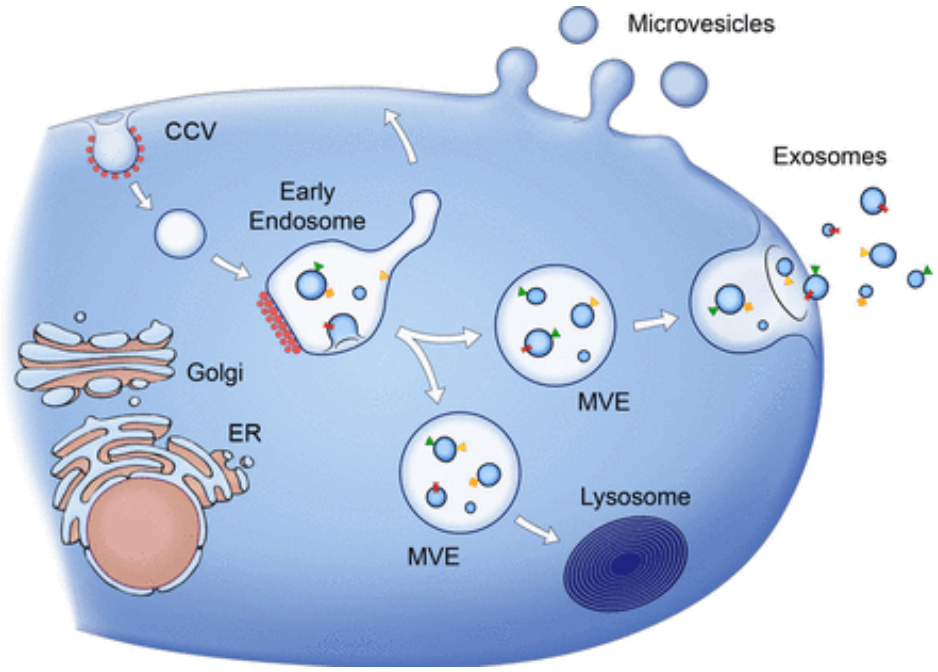


Figure 10. Extracellular vesicle formation. Extracellular vesicles: Exosomes, microvesicles, and friends. Raposo et. al. JCB 2013[411]

Extracellular vesicles have been demonstrated to contain proteins[412], particularly the tetraspanin molecules CD9, CD63, and CD81[413], key components of the endosomal network from which EVs arise. EVs also cytokines[414], microRNAs[415] and mRNAs[416] that are capable of being translated into protein in the recipient cell[417]. While the contents and surface makeup of EVs closely mimic the parental cell or tissue, miRNAs appear to be enriched in EVs, compared to in the cytosol. This could be explained by the observation that the RISC machinery that processes miRNAs has been localized to the surface of MVBs[418].

While some groups purport that extracellular vesicle contents are merely a random sample of the cytosol with no directed selectivity of their contents[411], there is mounting evidence that the miRNA content of EVs is distinct from the parent cell[419,420], lending

weight to the arguments that their loading is selective and that EV-miRNAs are functionally relevant mediators of inter-cellular communication[271,407,416,418]. Of concern, quantitative analysis of EV-miRNA content has demonstrated that individual vesicles contain extremely small numbers of miRNA molecules, and it has been concluded by some that this implies that EV-miRNA could not function stoichiometrically in cells taking up the EVs[421]. However other groups dispute this, demonstrating active EV-miRNA in recipient cells at the single cell level[422].

3.1.2 Functional relevance of EVs in health

The ubiquitous presence of EVs in healthy individuals suggests that they serve an important function, although this remains broadly unelucidated. Various biological roles have been proposed, including the disposal of harmful cellular waste products[423] and the transmission of elements of intercellular communication. EV mediated intercellular communication has been most thoroughly studied in immune cell communication, where EVs appear to serve important roles both in the delivery of pathogenic materials to antigen presenting cells (APCs)[424], and also a mechanism by which infected cells can stimulate immune cell responses in an APC dependent[425], and independent manner[426,427].

3.1.3 EVs in Cancer

EVs released by tumour cells are known as Tumour Extracellular Vesicles (TEVs). There has been a major effort in characterizing TEVs to determine if they can be exploited in the diagnosis or treatment of cancer. This effort is complicated by the fact that EVs isolated from diseased subjects contain EVs from both healthy and diseased tissues. Thus, more work has been published using EVs derived from tumour cells in culture.

While one study suggested that TEVs serve to prime immune cells towards anti-tumour responses[428], the majority of studies on the role of TEVs have demonstrated that their function, on balance, serves the tumour, with roles in the promotion of tumour growth[429] and proliferation[430], metastasis[431], and immune suppression[271,432]. More recently, TEVs have been demonstrated to be an important mediator of MDSC expansion[272,273,279,433–435]. Of relevance, the tumour suppressing MicroRNA miR34a, a target of p53, and carried in TEVs, has be shown to be crucially involved in regulating the expansion of MDSCs[286].

3.1.3.1. TEVs in AML

It has been demonstrated that AML cells release membrane bound extracellular vesicles[412,414,416,436], which transport microRNAs[415], mRNAs[416], cytokines[414] and tumour-derived proteins[412] to surrounding cells.

TEVs from patients with active disease carried blast markers and their levels correlated with the chance of achieving remission[414], and in another study, the blast count[437]. TEVs from patients with AML carried blast markers CD33, CD34, CD117 and TGFβ1 and suppressed allogeneic cytotoxic activity of NK cells and promoted TReg expansion[438]. AML cell line K562 secreted TEVs containing the pro-angiogenic miRNA miR92[439]. In an intriguing work by Jiminez et al, drug resistance AML cells secreted vesicles carrying anti-apoptotic proteins which were taken up by drug sensitive clones, suggesting TEVs represent a mechanism of the propagation of therapeutic resistance[440]. Finally, patient derived AML TEVs educated AML cell lines blasts to increase homing to the bone marrow of xenografted mice, suggesting a role in mediating AML cell export to the immune suppressive niche[441].

3.1.4 Technical aspects in EV research

3.1.4.1 EV isolation

The first technique historically used to isolate EVs is the ultracentrifugation of cell-free culture medium or patient serum or plasma. Centrifuging at 100,000–110,000 ×*g*, or at lower speeds with density gradient centrifugation[442], are the most-commonly used methods, these techniques being very inexpensive but unfortunately labour intensive and often co-isolating cell debris and other free proteins[443]. A further limitation is that these methods do not offer size separation.

In an effort to isolate vesicles based on size, several commercially available filtration based methods can isolating vesicles in under two hours; but can also isolate other protein complexes within the same size bracket of 100-350nm[443].

EVs may be precipitated out of solution by polyethylene glycol or other polymers, such is SystemBio's *ExoQuick*[444]. These precipitation methods have the advantage of speed (<2 hours, sometimes with an overnight incubation step), but can be non-specific due to the co-precipitation of other proteins and cell debris [445].

Membrane-based affinity methods, such as Qiagen's *ExoEasy*[446] can isolate EVs from serum and plasma or cell culture supernatant but do not distinguish EVs by size.

Finally, affinity methods based on capture of exosomes using peptides or antibodies are being used as specific methods. For instance antibodies against the tetraspanin CD63, enriched in EVs, have been used to successfully isolate EVs[447].

3.1.4.2 EV characterization

The morphology of isolated EVs is most usually characterized using electron microscopy (EM) or atomic force microscopy (AFM), demonstrating EVs have a rounded shape with lipid bilayers, which can sometimes appear cup-like due to the drying and freezing preparatory processes[448]. Nano-particle tracking analysis, such as NanoSight[449], which combines laser light scattering microscopy and a charge-coupled device camera, can complement EM, calculating the size distribution of isolated vesicles down to 30nm in size, based on the Brownian motion of vesicles in suspension in cell culture supernatant or body fluids[450,451].

While the lower limit of detection for convention Flow Cytometers is approximately 300nm, next generation high resolution flow cytometers have been developed, capable of detection micro-particles down to the 100nm size and utilizing standardized beads to accurately size and quantitate EVs in solution[452,453]. Furthermore, EVs can be stained with fluorescent antibodies to detect surface proteins, with or without mounting of EVs onto beads first[454]. The protocols need to be slightly modified to allow for un-bound antibody to remain in solution, as washing off unbound antibody would require further laborious ultra-centrifugation steps[455].

Once isolated, EV contents can be analysed in much the same way as cell contents can. EV proteins may be extracted utilizing standard cell lysis buffers and detected by immunoblotting[456,457] or mass spectrometry[458]. The tetraspanins CD81, CD9 and CD63 are often used as “housekeeping” proteins, to assess for equivalency of protein loading between samples[459].

RNA may be isolated from EVs and subjected to conventional RT-PCR or micro-array. The choice of RNA isolation is important because the RNA in EVs is mostly small RNAs, which may sometimes be lost in standard RNA isolation techniques[460]. Phenol-based techniques (TRIzol®) and Qiagen's RNeasy, miRNAeasy and exoRNeasy are often used and several techniques are compared in this comprehensive review[461].

3.1.5 EVs in diagnostics/prognostics

EVs are an attractive source of biomaterial for biomarker studies because they are abundant and easily accessible in blood and other bodily fluids such as urine[462], amniotic fluid and ascites[463], saliva[464] and even tears[465]. EVs could be used in the early diagnosis of cancers because content of the EV broadly mimics the parental cell (with some exceptions as previously described). While the majority of circulating EVs may arise from platelets, and not malignant cells, it is known that EVs from cancer cells often carry on their surface a set of antigens, such as CD19 and CD20 on B cell lymphoma EVs[466], and CD34 from AML EVs[437], that can aid the identification of TEVs.

While the use of circulating EVs as biomarkers of disease is still in its infancy and so far not in standard clinical use, several studies have delineated EV miRNA signatures of cancer, in ovarian[467], lung[468,469], prostate[470], colo-rectal cancers[469], myeloma[471] and AML[472]. Furthermore, in oeseopheal squamous cell carcinoma, a miRNA signature could predict prognosis[473].

3.1.6 EVs in therapy

Given the observation that TEVs carry tumour antigens and are capable of presenting them to T cells in an APC dependent[424,425] and independent[426,427] manner, it seemed a promising idea to use them to induce anti-tumour responses, as a form of vaccination. The advantages of using EVs as vaccines are that they are non-living and are not thought be able to engraft disease. Furthermore, they are easily obtained from patient's serum or plasma, where some tumours are inaccessible to clinicians. Despite several promising pre-clinical studies demonstrating proof of principle[474], there are been only a handful of early phase clinical trials, using EVs derived from ascites[475], or tumour antigen loaded[476], or native[477] patient derived dendritic cell EVs. The studies collective showed some promising immunological changes, but little in the way of clinical efficacy.

EVs are ideal candidates for the delivery of small RNAs (e.g. miRNAs[478,479]/siRNAs[480]), so called "suicide proteins"[481], and drugs[482] that are limited by a short half-life[407]. Advantages of this approach include the biological tolerability of these endogenous vehicles, and their abundance without the need for a lengthy manufacturing processes[483]. They can pass the blood brain barrier[480], and there is some evidence that EVs may be naturally targeted[484], or at least may be exogenously manipulated to be targetable, to specific tissues[480,482,485].

Lastly, given the weight of data demonstrating that TEVs have immune suppressing effects, it has been suggested that filtering out TEVs from the blood of cancer patients may improve their responses to other therapies[486].

3.2 Aims of this study

The aim of this study was to morphologically characterize AML cell line derived EVs, and determine if they carry known AML antigens. Furthermore, we sought to elucidate export of EVs to surrounding cells, and the effects of this export on the expansion of MDSCs *in vitro.*

3.3 Methods

Extra-cellular Vesicle (EV) Isolation

To prevent contamination with bovine exosomes present in FBS, MOLM-14 and THP-1 cells were cultured in media supplemented with exosome-depleted FBS, for all EV experiments. EVs were isolated from MOLM-14 and THP-1 cell culture supernatant using the Total Exosome Isolation Kit (Invitrogen, Camarillo, CA, USA), and in latter experiments with Exoeasy spin columns (Qiagen, Germany) as per manufacturers protocol. The wash fractions were retained as negative controls for Electron Microscopy.

Extra-cellular Vesicle Flow Cytometry (High Sensitivity Flow Cytometry)

Extra-cellular vesicles were isolated as before, and re-suspended in PBS or Exoeasy column elution buffer. Cells were run on a MoFlo® Astrios™ flow cytometer (Beckman-Coulter), capable of detecting particles as small as 100nm, and compared to standardized sized beads.

Extra-cellular Vesicle Electron Microscopy

Extra-cellular vesicles isolated as before, and re-suspended in PBS and stored at -20ºC until use. 20ul of re-suspended EVs were pipetted onto formvar coated, carbon coated, and glow-discharged 200 mesh nickel grids and were allowed to settle for five minutes. Next, the liquid was blotted off the grids using a Whatman #1 filter paper and the grids were then stained with 10 microliters of 2% Uranyl Acetate for 1.5 minutes. Following staining, liquid was blotted off the grids using a Whatman #1 filter paper and the grids were allowed to dry overnight. Images were collected using a JEOL 1400 Transmission Electron Microscope (JEOL USA Inc. 11 Dearborn Rd, Peabody, MA 01960).

Extra-cellular Vesicle Immunoblotting

Extra-cellular vesicles were isolated as before, and re-suspended in RIPA lysis buffer (1:100 RIPA buffer: Protease and Phosphatase Inhibitor) and centrifuged at 13300rpm for 15 mins. The resultant supernatant was collected and the EV debris pellet discarded. To calculate the total protein concentration in each lysate, 2.5µl of lysate was added to 1ml of Coomassie reagent and protein in measured using a spectrophotometer. 30µl of lysate were made up to 50µl of laemmli buffer and BME and boiled at 100°C for 5 minutes in a heat block. Equal amounts of protein were loaded into a 10% gel alongside 5µl ladder and 10µl laemmli buffer and BME into blank wells. Gels were run at 80v in running buffer, until the lowest band had reached the bottom of the gel. Gels were transferred onto PVDF membrane using a semi-dry transfer machine at 15V for 43 minutes. Membranes were subsequently blocked for one hour on a rocking platform in 5% milk solution in TBST. Membranes were washed for 3x20minutes on the rocking platform in TBST before 1:1000 dilutions of primary antibody (anti-MUC1-C (Thermo Scientific, Waltham, MA) or anti-c-Myc (Cell Signalling, Danvers, MA)) made up in 5% milk in TBST were added and membranes incubated overnight at 4°C. The next morning, the primary antibody was removed, membrane washed thrice as above, and the appropriate horseradish peroxidase-conjugated secondary antibody added in the same

manner as the primary. After one hour, the membranes were washed and activated using a 50:50 mixture of luminol and oxidizing reagent (GE Healthcare), before being developed on radiographic film. Equal loading of protein was demonstrated by stripping the membrane and developing with anti-CD63 antibody.

Extra-cellular Vesicle Export Assays

MOLM-14 cells were fluorescently labelled red with GranToxiLux (OncoImmunin, MD) and treated with SYTO® RNASelect™ Green Fluorescent cell Stain (Invitrogen, Camarillo, CA, USA) for 30 minutes, as per manufacturer's protocol. Cells were washed three times in PBS in co-cultured for four hours at 37ºC in a humidified 5% CO_2 incubator, with healthy donor PBMCs in a ratio of 1:10 tumour cells to PBMCs. After four hours, cells were stained with CD11b-APC-Cy7, HLADR-PE-Cy7 and CD33-PE. Using Flow Cytometry, tumour cells were excluded with the red dye, and the green SYTO dye (indicating exosomal mRNA) was quantified in the PBMCs.

In parallel, MOLM-14 cells were fluorescently labelled red with GranToxiLux (OncoImmunin, MD) and treated with SYTO® RNASelect™ Green Fluorescent cell Stain (Invitrogen, Camarillo, CA, USA) for 30 minutes, as per manufacturer's protocol. Cells were washed three times in PBS and then cultured overnight in RPMI supplemented with bovine exosome depleted FBS. EVs were isolated as described, protein was quantified by Bradford Assay EVs were confirmed for green fluorescence by flow cytometry. 10ng of EVs were added to healthy donor PBMCs and after 1 hour, EVs were washed off the PBMCs with PBS and PBMCs were assessed for green fluorescence using flow cytomtery. The presence of green fluorescence in PBMCs indicated PBMC uptake of AML EVs.

3.4 Results

AML cells release 200-300nM EVs into their extracellular environment

AML EVs were isolated using the *ExoQuick* precipitation technique, and analysed by flow cytometry, and compared to size standardized beads, demonstrating minimal debris and particles between 200-300nM in diameter (Figure 11E).

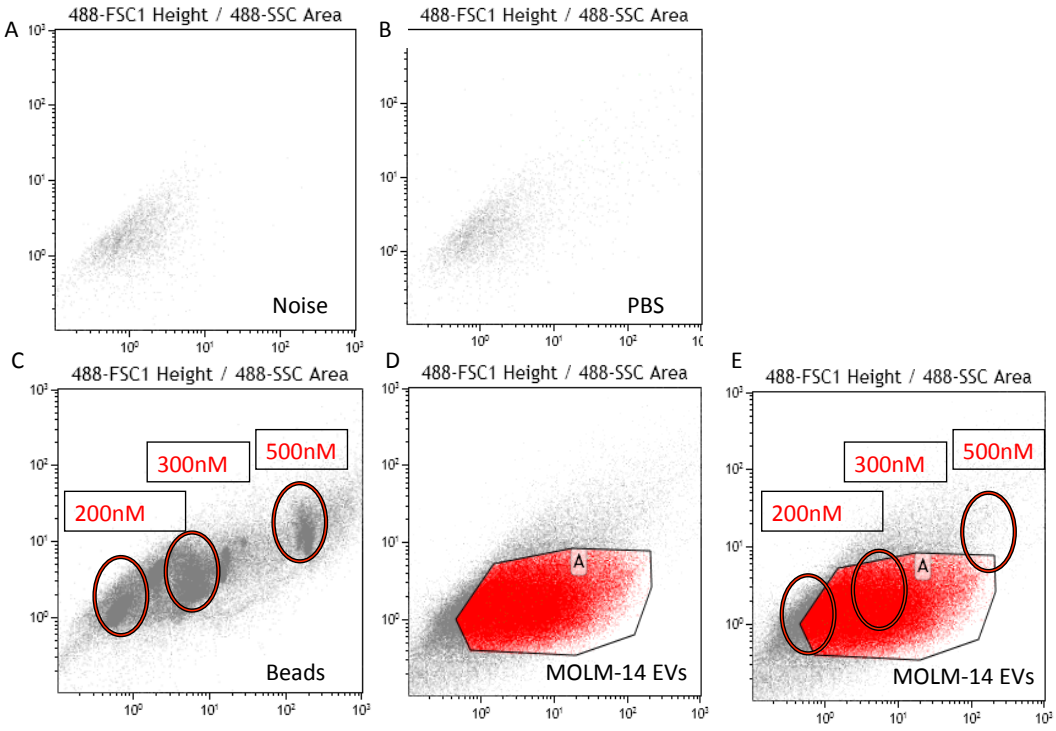


Figure 11. AML cells release 200-300nM vesicles into their extracellular environment

MOLM-14 AML EVs were isolated using a spin-column and characterized by flow cytometry. Background noise is shown in **(A)** and the negative control of PBS is shown in **(B).** A population of vesicles **(D)** were compared to sized beads alone in **(C),** and the gates super-imposed in **(E).** (n=3)

AML Extracellular Vesicles have a rounded morphology

MOLM-14 and THP-1 AML EVs were isolated using a spin-column and visualised using Transmission Electron Microscopy. The Uranyl Acetate staining highlights the thick glyo-lipid rich membrane and demonstrates multiple rounded structures measuring 100-200nM in diameter and bound by darkly staining membrane (Figure 12). Taken together we estimate the size of AML EVs to be around 200nm, as the dehydration step prior to electron microscopy may shrink EVs artificially[487], accounting for the difference seen between these modalities.

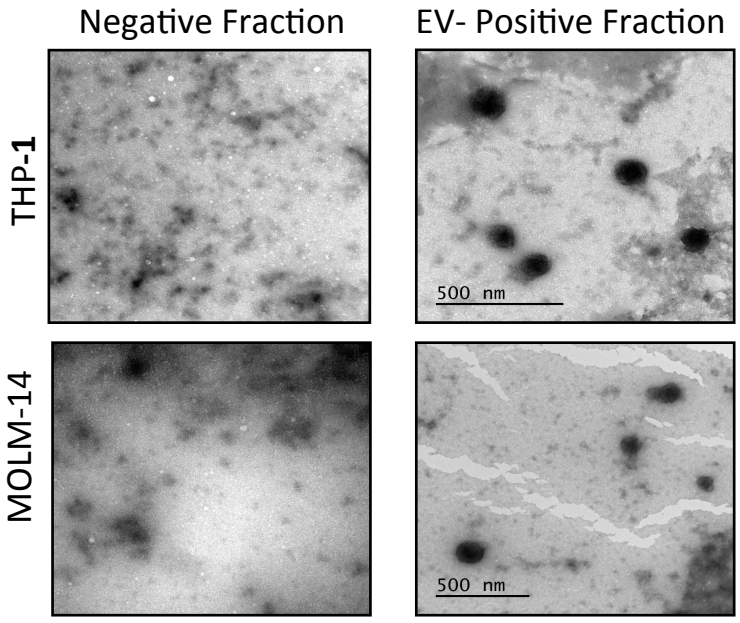


Figure 12. AML Extracellular Vesicles show a rounded morphology

MOLM-14 and THP-1 AML EVs were isolated using a spin-column and visualised using Transmission Electron Microscopy. The negative controls are the spin-column wash fractions, the elute being the vesicle-rich fraction. (n=2)

AML Extracellular Vesicles contain MUC1 and c-Myc protein

MOLM-14 and THP-1 AML EVs were isolated using a spin-column, and lysates were subjected to immunoblotting for the AML antigens MUC1 and c-Myc. Figure 13 shows that the EV lysates contained MUC1 and c-Myc proteins, and stained strongly for the EV associated tetraspanin protein CD63.

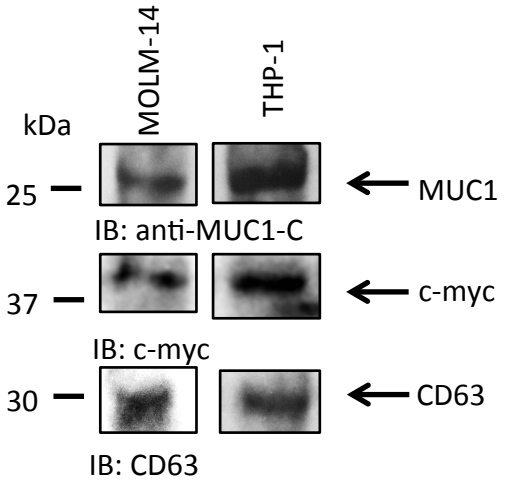
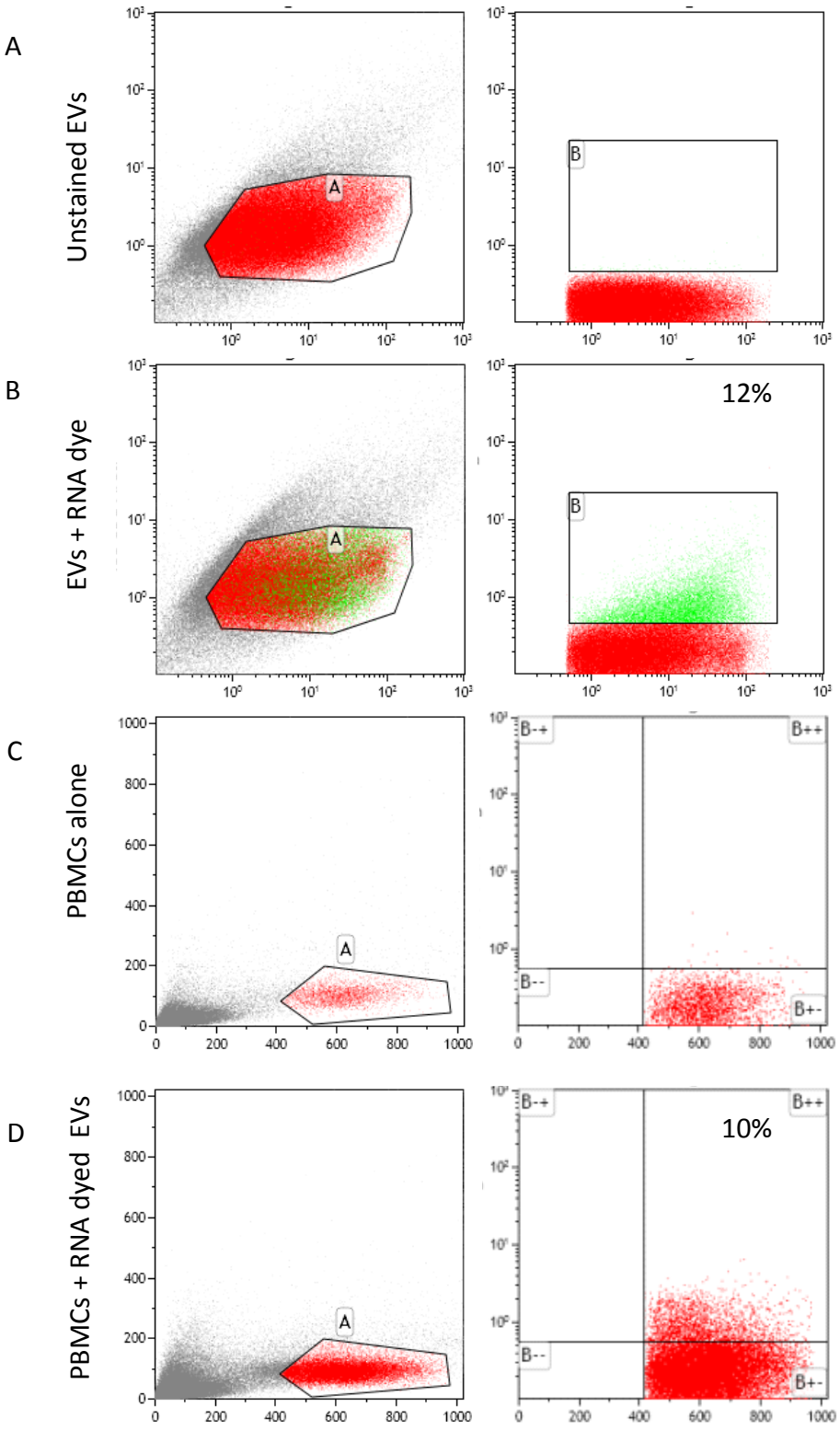
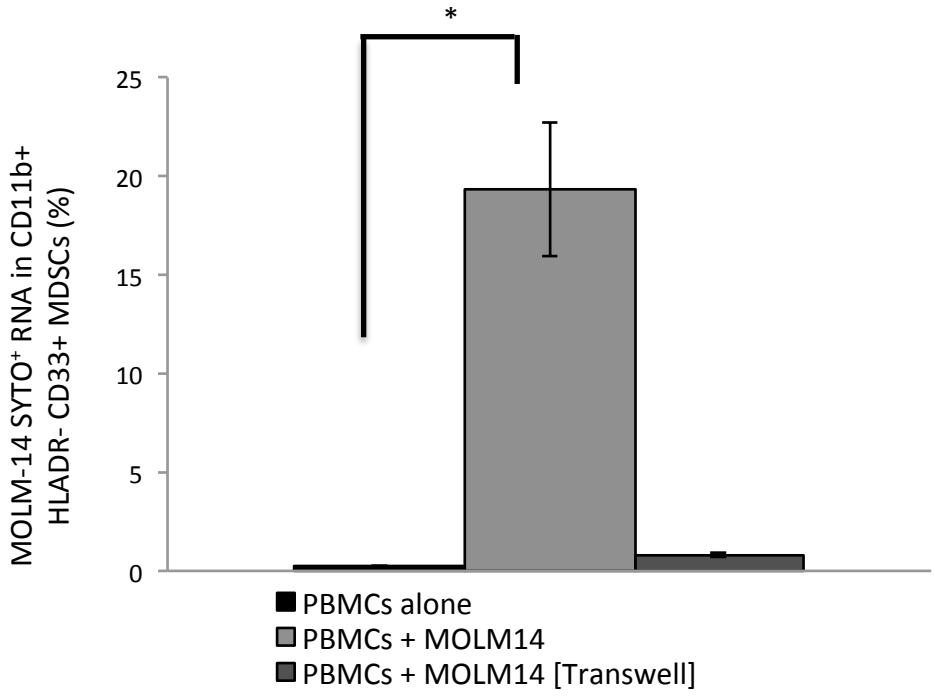


Figure 13. AML Extracellular Vesicles contain MUC1 and c-Myc protein

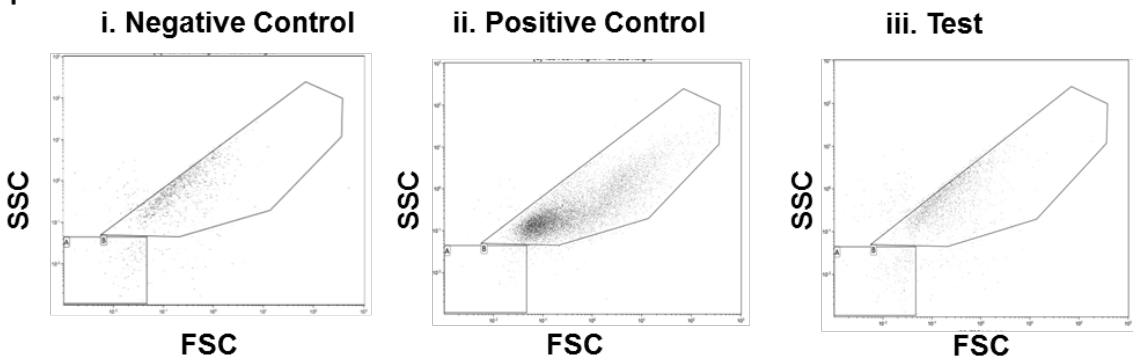
MOLM-14 and THP-1 AML EVs were isolated and lysed before immunoblotting for the oncoproteins MUC1 and c-Myc, and the EV associated tetraspanin protein CD63. (n=2)


A
Unstained EVs
A
B
B
EVs + RNA dye
12%
A
B
C
PBMCs alone
A
B-+
B++
B--
B+-
D
PBMCs + RNA dyed EVs
10%
A
B-+
B++
B--
B+-

E



F



Transwell	i.	ii.	iii.
Top chamber	RPMI	RPMI	EVs
Bottom Chamber	RPMI	EVs	RPMI

	i.	ii.	iii.
EV Count (Events/30 seconds)	5643	87961	5395

Figure 14. AML Extracellular Vesicles export to surrounding cells, including MDSCs

MOLM-14 AML cells were treated SYTO RNA dye or control. EVs were isolated as before and assessed for SYTO fluorescence, indicating AML RNA, by flow cytometry. Unstained EVs are shown in **(A)** and EVs from SYTO treated AML cells are shown in (n=2) **(B).** Healthy donor PBMCs were co-cultured with SYTO stained EVs. After six hours, PBMCs were assessed for the presence of SYTO fluorescence, indicating uptake of AML RNA, by flow cytometry **(C and D)** (n=3)**.** Subsequently, to determine if AML EVs export to surrounding cells, PBMCs were co-cultured in direct contact or in Transwell, with MOLM-14 AML cells pre-treated with SYTO RNA dye (530nm). After six hours, MDSCs were quantified for AML SYTO RNA dye using flow cytometry. Summary of three experiments is shown in (n=3) **(E).** EVs were isolated from cell culture supernatant as previously described. (F) 0.4uM Transwells were set up as shown and incubated for 3 hours at room temperature. Thereafter, 400ul of RPMI was sampled from the bottom chamber and subjected to high sensitivity Flow Cytometry, whereby EV count was quantified as events per 30 seconds, and tabulated as shown. (n=3)

AML Extracellular Vesicles export to surrounding cells, including MDSCs

Healthy donor PBMCs were co-cultured with RNA-dyed stained EVs (SYTO dye). After six hours, PBMCs were assessed for the presence of SYTO fluorescence, indicating uptake of AML RNA, by flow cytometry. Figure 14E demonstrate 18% of PBMCs contained SYTO stained EVs after 6 hours, indicating EV export and uptake by surrounding cells. To confirm that EV export is abrogated in Transwell, purified EVs were placed in the top compartment of a Transwell well and sterile RPMI in the bottom chamber. After three hours, the RPMI in the bottom chamber was collected and subjected to high sensitivity Flow Cytometry, demonstrating no EVs in the bottom compartment (Figure 14F).

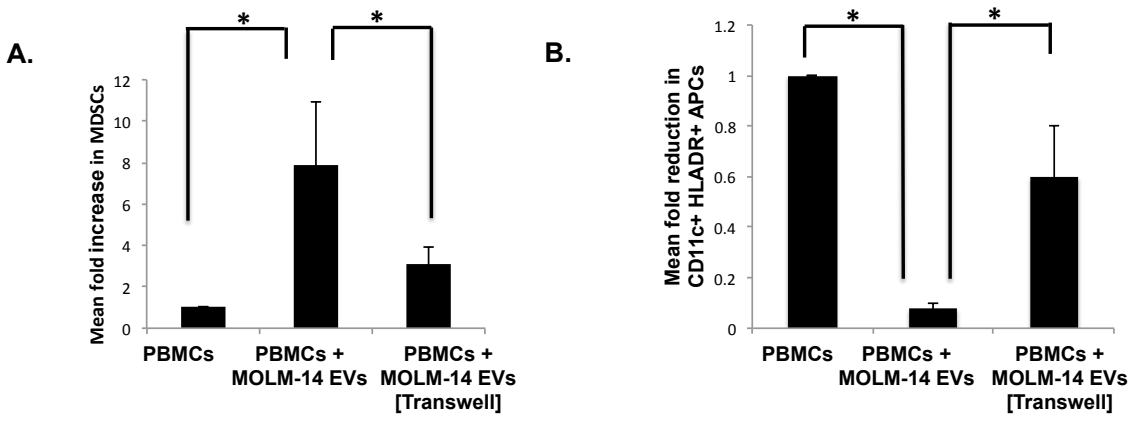


Fig 15. AML Extracellular Vesicles alter the tumour microenvironment. Healthy donor PBMCs were cultured for three days with AML EVs, in direct contact or in Transwell, and then quantified for **(A)** CD11b+/HLADR-/CD33+ MDSCs (expressed as a percentage of immature CD11b+/HLADR- myeloid cells) and **(B)** HLADR+/CD11c+ Myeloid APCs by flow cytometry (n=3, $p<0.05$).

AML Extracellular Vesicles alter the tumour microenvironment

Healthy donor PBMCs were cultured for three days with GFP tagged AML EVs in direct contact or in Transwell, and then quantified for CD33+ HLADR-, CD11b+ MDSCs (Figure 15A) and HLADR+ CD11c+ Myeloid Antigen Presenting Cells (APCs) (Figure 15B) by flow cytometry. In the PBMCs co-cultured with EVs, the proportion of MDSCs increased 8-fold, whilst the proportion of HLADR+ CD11c+ Myeloid APCs decreased by 10-fold. Absolute frequencies of cells are shown in Appendix F.

3.5 Discussion

These data collectively demonstrate that MOLM-14 and THP-1 AML cell lines secrete extra-cellular vesicles. These vesicles had a size of between 200-300nM when measured by Flow Cytometry, and of 100-200nM when measured by Electron microscopy. This disparity is in keeping with published comparisons between measuring modalities[488], which suggest that flow cytometry may be a slightly more accurate method of measurement, given the changes to vesicle structure upon dehydration, fixation and freezing for electron microscopy. In this study, we can conclude that vesicles probably have a size distribution around 200nM, with THP-1 EVs appearing slightly larger than MOLM-14 EVs (Figure 12), consistent with the larger size of the parent THP-1 cell, compared to MOLM-14.

Thereafter, we have demonstrated that TEVs contain the AML oncoproteins MUC1 and c-Myc. This observation is important as it suggests that AML EVs may be pro-tumourigenic and immune suppressive, by carrying these well-studied oncoproteins to surrounding cells. Conversely, AML EVs may serve to educate nearby immune cells by presenting tumour-associated antigens, capable of eliciting an anti-tumour immune response. Lastly, this observation offers the possibility of AML EVs being used *ex vivo*, in the manufacture of anti-tumour vaccines, with EV oncoproteins priming dendritic cells against tumour cells.

To demonstrate that AML EVs are not only secreted by AML cells, but are also capable of being taken up by nearby cells, we sought to elucidate export of EVs to surrounding cells. Utilizing a RNA-specific fluorescent dye, we demonstrated that RNA containing AML EVs export to, and are taken up by, co-cultured PBMCs. Importantly, we demonstrated that EV passage is abrogated in 0.4uM Transwell, in keeping with

previous studies[406,407], and hypothetically due to EVs aggregating into larger particles which are sterically hindered from traversing the Transwell pores.

Finally, to elucidate the effect of AML EVs on surrounding cells, flow cytometry for markers of immature myeloid cells (MDSCs) and mature myeloid cells (DCs) was performed on PBMCs treated with AML EVs. After three days of EV-PBMC co-culture, there was an increased proportion of cells carrying the MDSC markers (CD33+, HLADR-, CD11b+) and a statistically significant decrease in cells carrying markers of a mature myeloid phenotype (HLADR+ CD11c+, putatively antigen presenting cells/DCs). This suggests that AML EVs induce a skewing of their surrounding cells, from a mature to an immature and suppressive myeloid phenotype.

The mechanism by which this skewing occurs remained to be elucidated. We hypothesized that AML cells may promote the expansion of MDSCs by inducing immature myeloid cells to proliferate, thereby undergoing a differentiation block. This would be in contrast to normal myeloid cell development, characterized by the arrest of proliferation and their differentiation into terminal myeloid cells such as macrophages and dendritic cells.

We hypothesized that AML cells may release in EVs, an element such as a protein, a miRNA, or set of miRNAs, that promotes this proliferation of immature myeloid cells. Given the widely reported similarity in content of EVs to their parental cell line, and the significantly increased ease in studying cell contents instead of EV content, we started by investigating proteins and miRNAs in AML cells that may promote MDSC expansion.

Mucin 1 (MUC1) is a heterodimeric protein that regulates critical pathways of

oncogenesis including those governing cell proliferation, self-renewal, tissue invasion, and apoptosis[8]. MUC1 has been identified as a uniquely important oncoprotein in AML and AML stem cells that exerts immune-modulatory effects[8]. It has been demonstrated that MUC1 is selectively expressed on AML stem cells as compared to normal haematopoietic stem cells, and is critically involved in the self-renewal capacity of malignant cells[128]. In this study we had observed that MUC1 was present in AML EVs. Given the critically important role of MUC1 in AML, and its immune suppressive effects, we hypothesized that MUC1 expression in AML cells may promote the expansion of MDSCs seen. The next chapter details the investigations testing this hypothesis.

Chapter 4. MDSC expansion is dependent on AML expression of MUC1

4.1. Introduction

A series of murine and human *in vitro* and *ex vivo* studies suggest that tumour cells secrete factors into the microenvironment that promote MDSC expansion. In human studies, tumour-conditioned medium has been used to expand MDSC-like cells from peripheral blood mononuclear cells[256] with GM-CSF an IL-6 proving critical mediators of this expansion.

In murine studies, tumour-conditioned medium expanded MDSC-like cells from bone marrow mononuclear cells[257] or splenocytes[258], with GM-CSF being repeatedly reported to be the key inflammatory mediator[259–261]. Other implicated cytokines in murine MDSC expansion include M-CSF[262], G-CSF[263], SCF[264], CSF[265], IL-6[266], Il-1B[267] and TNFalpha[268].

The expansion and regulation of MDSCs is not solely dependent on soluble cytokine type factors, however.

Key signalling pathways Involved in MDSC Expansion

The Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway has a critical role in mediating both the expansion of MDSCs and their function in suppressing immune cells[274–276]. Unsurprisingly, inhibitors of STAT activation have been used to attempt to target this population, as we will later discuss. STAT3[277] and STAT5 have key roles in MDSC expansion, putatively via their roles in inflammatory cytokine production[278]. STAT3 inhibition has been shown to mediate differentiation of MDSC into mature Dendritic Cells, indicating aberrant STAT3 signalling plays an important role in cancer, in maintaining myeloid cells in an immature, more immune-suppressive state[274,279]. In head and neck cancers STAT3 was shown to control MDSC function by regulating Arg-1 activity[280]. MDSCs from tumour bearing mice have high levels of activated STAT3[281]. The inhibition of STAT3 with the tyrosine kinase inhibitor Sunitinib, blocked expansion of MDSC in tumour bearing mice[282]. Consistent with this finding, activation and overexpression of STAT3 in myeloid cells led to an expansion of MDSC in

murine model of lung cancer[283]. STAT3 activation up-regulated the pro-inflammatory protein S100A8/9, which inhibits the differentiation of DCs and macrophages, leading to an accumulation of MDSCs[210]. There is an inverse correlation between the percentage of granulocytic MDSCs and levels of STAT1 phosphorylation in CD4 T cells. Co-culture of MDSCs and CD4 T cells from healthy donors led to reduced IFN-y responsiveness[284]. STAT6-deficient MDSCs fail to inhibit T cell activation as they failed to up-regulate iNOS or make Arg-1[285].

The transcription factor Twist is associated with diverse malignancies. In one study, overexpression of Twist in cancer cell lines was associated with an expansion of murine MDSCs in co-cultured myeloid precursor cells. Conversely, siRNA silencing of Twist expression partly abrogated the expansion of MDSCs in this model[286].

The RAS signalling pathways are key regulators of normal cell growth and malignant transformation[287]. Ras signalling plays a key role in myeloid development and promotes granulopoeisis, and thereby the production of granulocytic MDSCs, by increasing the binding of C/EBP alpha to the GCSF receptor[288]. Overexpression of kRas in a murine model of pancreatic cancer cells led to increased expression of the chemoattractant cytokines MIP-2 and MCP-1, which promote the recruitment of macrophages and MDSCs into the tumour microenvironment[289].

PI3K/Akt signalling affects cell growth, survival migration and metabolism that is thought to play a significant role in MDSC expansion. Aging mice accumulate MDSCs in their BM and secondary lymphoid organs which was demonstrated to be related to a PI3K/AKT signalling defect in MDSCs [290]. Moreover, SHIP and PTEN proteins are negative regulators of PI3K signalling[291] and a dramatic increase in MDSCs is seen in SHIP knock out mice[292].

MicroRNAs Involved in MDSC Expansion

An alternate mediator of oncogenesis is the presence of microRNAs that bind and degrade specific mRNAs disrupting translation and creating post-transcriptional effects that promote the malignant phenotype.

In one study, the microRNAs miR155 and miR21 were the two most upregulated miRNAs during the induction of MDSC from the bone marrow cells by GM-CSF and IL-6 and targeting these miRNAs abrogated cytokine induced MDSC expansion *in vitro*[293]. miR155 was further demonstrated to mediate tumour induced MDSCs in a murine models of lymphoma[294], and breast cancer[295].

miR34a targets p53 and has been linked to the expansion of MDSCs. The combination of Twist silencing and miR34a overexpression nearly completely abrogated the expansion of MDSCs[286].

Other Factors Promoting MDSC Expansion

Hypoxia is a common feature of solid tumours as they outgrow their blood supply[296], and has implicated in mediating resistance to chemotherapy[297,298], the promotion of metastases[299], as well as resulting in an immune privileged niche[93,300–302]. Hypoxia has been shown to promote the expansion of immune-suppressive MDSCs[303,304], as well as mediating the differentiation of MDSCs into immune-suppressive tumour associated macrophages (TAMs), upon arrival in the hypoxic tumour bed[302].

While the presence of tumours has been clearly demonstrated to result in the recruitment and proliferation of MDSCs, various cancer treatments have also been implicated in further expanding this population. Several cytotoxic chemotherapeutic agents including cyclophosphamide[305,306], doxorubicin[234] and melphalan have been associated with an expansion of MDSC-like cells, although in one study these “induced MDSC” were not as immune-suppressive as native MDSC[307]. It is worth noting that GM-CSF, widely used as an immune adjuvant in cancer vaccines, is the most reported MDSC

promoting cytokine, although evidence of MDSC expansion in GM-CSF containing treatments remains conflicting[308].

While we have divided MDSC inducing factors into discrete sections, it is important to note that there is a huge amount of cross-talk and overlap in these pathways, with transcription factors mediating cytokine release, and miRNAs, such as miR155, implicated in promoting transcription factor STAT3 activity[277].

In our experiments, we observed an apparent need for cell-cell proximity between tumour and co-cultured PBMC, in order to elicit MDSC expansion. This is in contrast to the previous research in solid tumours that implicated soluble factors such as cytokines in MDSC expansion. It might not be surprising that the mechanism of MDSC expansion relies on different factors in solid and haematological malignancies, given the enormous differences in the tumour bed and pathogeneses of these different malignancy groups. We hypothesized the surface expression (and in parallel the EV expression) of a tumour antigen might be integral in the mechanism of MDSC expansion in AML.

The MUC1 oncogene is aberrantly expressed in solid tumours[131] and haematologic malignancies including AML[128] and plays a critical role in maintaining the malignant phenotype[489–491]. Signalling via the MUC1-C subunit supports tumour cell proliferation and resistance to apoptosis[492]. Recent studies have suggested that MUC1 demonstrates immunoregulatory properties[493]. As such, we investigated whether MUC1 exerts an immunosuppressive effect on the tumour microenvironment by inducing the expansion of MDSCs.

4.2. Aims of the study

To test this hypothesis, we sought to generate AML cell lines silenced for the expression of MUC1-C, the active cytoplasmic domain of the MUC1 molecule[30]. Thereafter, we would repeat the co-culture experiment, incubating MUC1 silenced AML cells with healthy donor PBMCs, to elucidate if MUC1 silencing would diminish MDSC expansion *in*

vitro. If this hypothesis proved correct, we would then aim to delineate the signalling pathway underlying this observation.

4.3. Materials and Methods

Generation of MUC1 silenced AML cell lines

There are several methods utilized to silence the expression of proteins. We utilized two such methods, lentiviral transduction of shRNA and CRISPR/Cas9 technology.

Lentiviral transduction of shRNA employs a lentivirus, capable of integrating its genetic material into that of the infected cell, and delivering a small hairpin RNA (shRNA) which can bind to and target the translation of specific mRNA. The integrated genetic material is passed onto progeny cells, creating a population of cells stably silenced for a protein of interest. To select for cells that have been successfully transduced with the lentivirus, the genetic package often includes a gene conferring resistance to a particular antibiotic. Thus, culturing transduced cells in that antibiotic will kill all un-infected cells resulting in a pure population of successfully silenced cells[31].

The human AML cell lines THP-1 and MOLM-14 and the murine AML cell line TIB-49 cells were transduced with lentiviral vectors expressing MUC1-C, or control MUC1-C(AQA) shRNA (Sigma, St. Louis, MO) and maintained in 2μg/ml Puromycin (Sigma, St. Louis, MO) to select for successfully transduced clones.

In addition, CRISPR/Cas9 technology was employed. CRISPR systems are adaptable immune mechanisms used by many bacteria to protect themselves from foreign nucleic acids, such as viruses or plasmids, by incorporating them into their own genome. The addition of a sequence of DNA corresponding to the gene of interest, directs Cas9 to cleave complementary target DNA sequences. Thus, with this system, Cas9 nuclease activity can be directed to 'chop out' any particular DNA sequence.[32] For the CRISPR edited cell line, sgRNAs targeting the first exon of the MUC1 gene were cloned into a

lenti-plasmid (Genome Engineering Production Group, Harvard Medical School). MOLM-14 cells were transduced with viral vector containing the lenti-CRISPR plasmid and successfully transduced clones were selected for by limiting dilution and maintained in 2µg/ml Puromycin (Sigma, St. Louis, MO).

Cytokine Array

In order to assess MUC1 silenced cells for difference in cytokine production, cytokine array was performed on cell culture supernatants of MUC1 silenced and control MOLM-14 and THP-1 AML cells. Cytokine array comprises incubation of cell supernatant, lysate or plasma on a membrane impregnated with antibodies against an array of cytokines. Thereafter, the membrane is incubated with a fluorescent secondary antibody and them membrane developed to result in dots of varying intensity of density, reflecting the concentration of cytokines in the sample.

AML cells at a concentration of $1x10^6$ cells/ml of complete media were cultured at 37ºC in a humidified 5% CO_2 incubator for 24 hours in the presence of 1µg/mL LPS (Sigma, St. Louis, MO), in order to stimulate cytokine secretion. Supernatant was collected by centrifuging the culture twice at 1600rpm and frozen at -20°C until use. Cytokine array was performed using Human Cytokine array Kit, Panel A, as per manufacturer's protocol (R&D, Minneapolis, MN).

Western Blotting

Whole cell lysates were prepared by sonicating pellets of at least $1x10^6$ cells in RIPA lysis buffer (1:100 RIPA buffer: Protease and Phosphatase Inhibitor) and centrifuged at 13300rpm for 15 mins. The resultant supernatant was collected and the cell debris pellet discarded. To calculate the total protein concentration in each lysate, 2.5µl of lysate was added to 1ml of Coomassie reagent and protein in measured using a spectrophotometer. 30µl of lysate were made up to 50µl of laemmli buffer and BME and boiled at 100°C for 5 minutes in a heat block. Equal amounts of protein were loaded

into a 10% gel alongside 5µl ladder and 10µl laemmli buffer and BME into blank wells. Gels were run at 80v in running buffer, until the lowest band had reached the bottom of the gel. Gels were transferred onto PVDF membrane using a semi-dry transfer machine at 15V for 43 minutes. Membranes were subsequently blocked for one hour on a rocking platform in 5% milk solution in TBST. Membranes were washed for 3x20minutes on the rocking platform in TBST before 1:1000 dilutions of primary antibody (anti-MUC1-C (Thermo Scientific, Waltham, MA)) made up in %% milk in TBST were added and membranes incubated overnight at 4°C. The next morning, the primary antibody was removed, membrane washed thrice as above, and the appropriate horseradish peroxidase-conjugated secondary antibody added in the same manner as the primary. After one hour, the membranes were washed and activated using a 50:50 mixture of luminol and oxidizing reagent (GE Healthcare), before being developed on radiographic film. Equal loading of protein was demonstrated by stripping the membrane and developing with anti-GAPDH or B-actin antibody.

Quantitation of MicroRNA and mRNA by qPCR

For detection of miR34a and pre-miR34a miRNAs from total RNA, the QuantiMir Small RNA Quantitation System (System Biosciences) was used as per the manufacturer's protocol. For detection of Dicer1, cJUN and c-Myc mRNA, total RNA was extracted using the RNeasy system (Qiagen, Germany). cDNA was manufactured using ThermoFishers SuperScript VILO Mastermix (ThermoFisher, Waltham MA) and RT-PCR was performed. The qPCR forward primers for detection of miR34a and pre-miR34a miRNA, and the DICER1, cJUN and c-Myc mRNA, are listed in Appendix C and the universal reverse primer was supplied in the detection kit. B-actin or GAPDH were used to confirm cDNA synthesis in mRNA PCR, and 18s for miRNA PCR. Statistical significance was determined by the Student *t* test.

Generation of miR34a overexpressed AML cell lines

AML cells were infected with lentiviral vectors expressing a GFP-miR34a[33] mimic (pLL3.7_hsa-miR34a which was a gift from Judy Lieberman (Addgene plasmid # 25791)) or a scrambled control vector and successfully transfected cells were isolated using FACS for GFP positive cells, and expanded in culture.

Generation of miR34a silenced AML cell lines

miR34a was stably silenced in in MUC1 silenced AML cells by lentiviral transduction of a miRZip-34a anti-miR34a microRNA construct (SystemBio, CA). Successfully transduced cells were isolated by FACS for GFP positive cells.

Treatment with MUC1-C inhibitor

AML cells were treated daily with varying doses of the MUC1-C inhibitor "GO-203" to generate dose curves (see Appendix D). Apoptotic and dead cells were measured using Flow Cytometry staining for Annexin V and Propidium Iodide (ThermoFisher, Waltham MA). The highest sub-lethal dose (defined as <10% dead or apoptotic cells) of GO-203 was determined to be 1uM for MOLM-14 and THP-1. AML cells were treated with 1uM GO-203 or the inactive control compound CP3, daily for 72 hours. After 72 hours cells were harvested, washed twice in RPMI, pelleted and lysed in RIPA buffer for further analysis by Western Blot.

mRNA Array

RNA was isolated from paired GO-203 treated and untreated AML samples from 3 subjects. Affymetrix gene array for small RNAs was performed as per manufacturer's protocol and all arrays were included in the differential expression analysis. Standard normalization methods were applied, followed by paired T-test analysis for Bonferroni-corrected significance, using Limma in Bioconductor software.

miRNA Array

RNA was isolated from AML cells as described and run in triplicate on a NanoString (Seattle, WA) nCounter instrument using the human miRNA Expression Assay Kit v3, according to the manufacturer's instructions. Data obtained were then normalized to positive miRNA-ligation reaction controls and background noise was subtracted. Statistical significance was determined by T-test analysis for Bonferroni-corrected significance, and fold change of miRNAs was calculated.

4.4 Results

MUC1 is important in the expansion of MDSCs

To elucidate if the membrane onco-protein MUC1 might be responsible for the direct cell contact required expansion of MDSCs, stably transduced cell lines silenced for the expression of MUC1 protein were generated by lentiviral transduction of shRNA or control vector (Figure 16 A), or CRISPR/Cas9 technology (Figure 16 B). Healthy donor PBMCs co-cultured in direct contact with irradiated MUC1 silenced AML cells resulted in a 60% reduction in expansion of MDSCs compared to control AML cells, in MOLM-14 and THP-1 cells lines (Figure 16 C and D). Absolute frequencies of cells can be found in Appendix F.

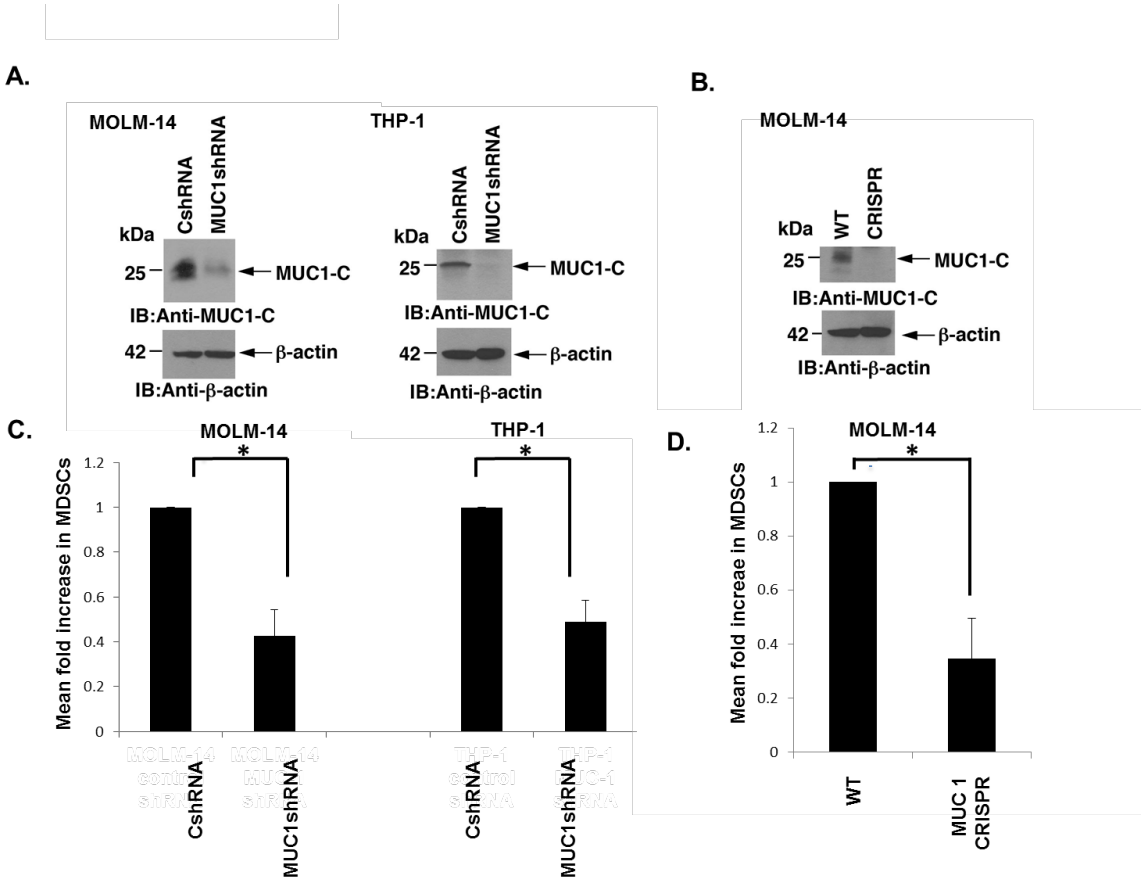


Figure 16. MUC1 is critical in the expansion of MDSCs. Stable MOLM-14 and THP-1 AML cell lines silenced for the expression of MUC1 protein were generated by lentiviral transduction of shRNA or control vector. (n=2) **(A)** Lysates were prepared and cells analysed for MUC1 expression using Western blotting. To validate the silencing, MOLM-14 cells were silenced for MUC1 expression using CRISPR/Cas9 technology (n=2) **(B).** Lysates were prepared and cells analysed for MUC1 expression using Western blotting using β-actin as a loading control. Healthy PBMCs and irradiated, fluorescently labelled MUC1 silenced and control AML cells were co-cultured for five days at a ratio of 100:1 (PBMC:AML). After co-culture, cells were analysed by Flow Cytometry and fluorescently labelled blast cells were excluded, CD11b+/HLADR-/CD33+ MDSCs were quantified as a percentage of immature CD11b+/HLADR- myeloid cells. Summary of three independent experiments for **(C)** MUC1 silenced MOLM-14 and THP-1 AML cells, and **(D)** CRISPR/Cas9 MUC1 silenced MOLM-14 cells are shown (n=3, $p<0.05$).

The cytokine profile of MUC1 silenced AML cells is unchanged

To elucidate if MUC1 silencing had an effect on the production of cytokines implicated in MDSC recruitment, cytokine array of MUC1 silenced and control AML cells was performed on cell culture supernatants from MOLM-14 (Figure 17 A) and THP-1 (Figure 17 B) AML cells. There was no significant difference seen in any secreted cytokine upon MUC1 silencing, notably GM-CSF, IL-1β and IL-6, implicated in MDSC recruitment.

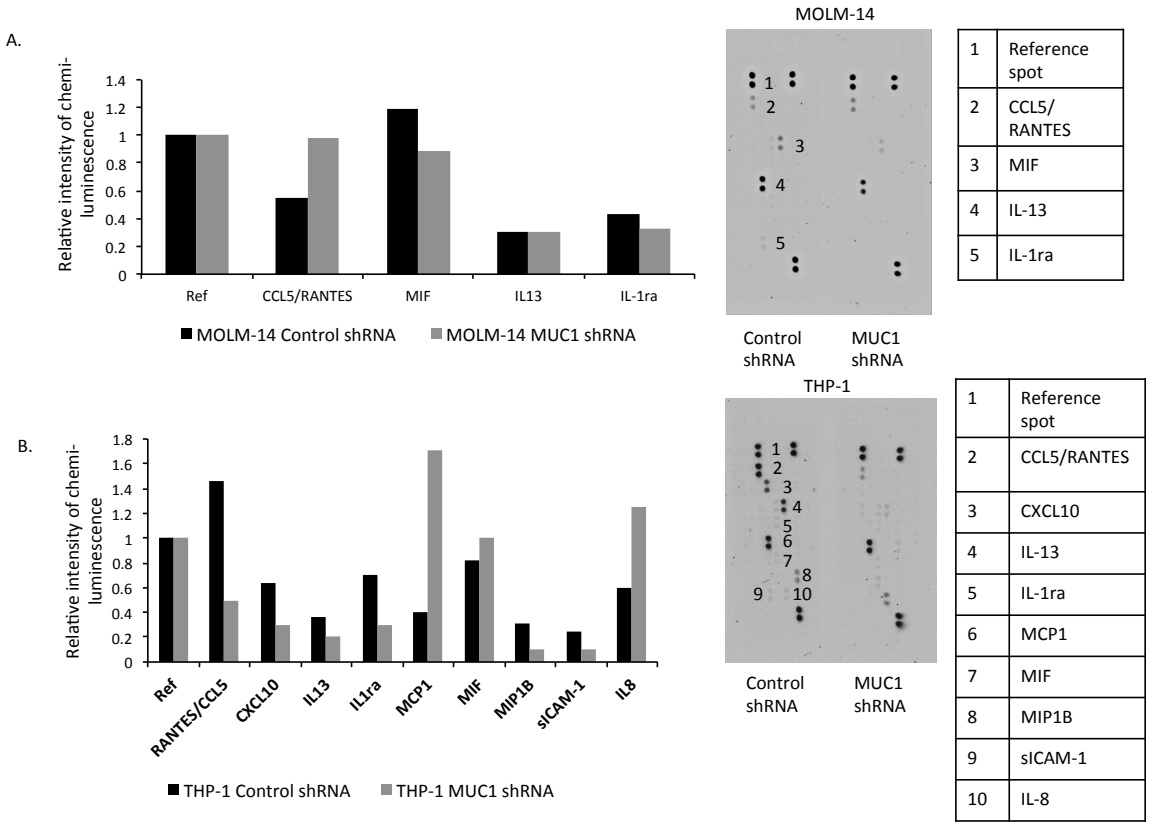


Figure 17. Cytokine array of AML cells is grossly unchanged in MUC1 silenced cells.

MOLM-14 and THP-1 AML cells were silenced for MUC1 expression using specific MUC1 shRNA, or control shRNA. Cells were stimulated for 24 hours with LPS and supernatant collected. Cytokine array was performed on cell culture supernatant. Representative histogram and membranes are shown of (n=2) experiments on **(A)** MOLM-14 and **(B)** THP-1 AML cells.

We sought to elucidate a soluble factor independent mechanism by which MUC1 might be inducing the expansion of MDSCs.

MUC1 mediates tumour cell proliferation via downstream effectors including prominently the oncoprotein, c-Myc that regulates expression of cell cycle proteins[489,490]. However, the role of oncoproteins as immunoregulatory agents that mediate MDSC expansion in the tumour microenvironment has not been elucidated. We postulated that MUC1 mediated expression of c-Myc in AML cells would potentially

impact proliferation of MDSCs in the tumour microenvironment through its transfer via AML derived EVs.

The c-Myc protein is nuclear phospho-protein that plays a critical role in cell cycle progression and proliferation[494]. Recent studies in myeloma and lung cancer have demonstrated that MUC1 signalling promotes c-Myc expression[495,496]. We sought to elucidate if MUC1 signalling promotes c-Myc expression in AML, and moreover, if these proteins are exported in EVs to nearby MDSCs, inducing a proliferative expansion.

AML MUC1 signalling drives c-Myc expression in AML cells and Extra-cellular Vesicles

MUC1 mediates tumour cell proliferation via downstream effectors including prominently the oncoprotein, c-Myc. Of note, EVs derived from MUC1 expressing AML cells demonstrate the presence of MUC1 and c-Myc as determined by Western blot analysis (Figure 18B). Furthermore, silencing of MUC1 in the parent AML cell lead to a reduction of MUC1 and c-Myc in the cells and EVs derived from those cells (Figure 18 A and B).

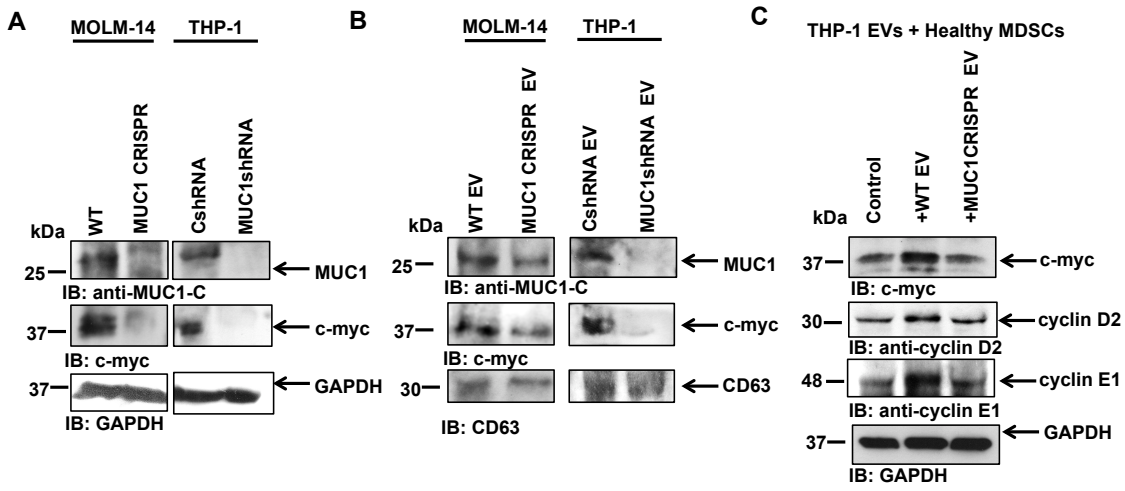


Fig 18. MUC1 promotes c-Myc expression in extracellular vesicles, which leads to up-regulation of cyclin D2 and E1 in co-cultured MDSCs. Stably transduced cell lines silenced for the expression of MUC1 protein were generated by lentiviral transduction

of shRNA or CRISPR/Cas9 technology. Control shRNA or wildtype cells were used as controls. Lysates were prepared and MUC1 and c-Myc expression was assessed using western blot analysis in **(A)** AML cells (n=3), and **(B)** in isolated secreted EVs generated from MOLM-14 and THP-1 cells (n=2). GAPDH and CD63 were used as loading controls. CD11b+/HLADR-/CD33+ MDSCs were isolated from healthy donor PBMCs and cultured for 48 hours with EVs isolated from the culture medium of THP-1 cells, PBMCs were lysed and **(C)** subjected to immunoblot for c-Myc and Cyclins D2 and E1. GAPDH was used as loading control.(n=1).

MDSCs exposed to AML-EVs have increased c-Myc and c-Myc dependent downstream mediators of proliferation

To determine if the export of c-Myc containing EVs to myeloid cells in the microenvironment resulted MDSC expansion, MDSCs were pulsed with EVs from wild type or MUC1 silenced AML cells. Figure 18 C shows that control EV treated MDSCs contained increased levels of c-Myc and increased expression of cyclin E1 and cyclin D2, downstream pro-proliferative targets of c-Myc. Cyclin D1 did not change. MDSCs exposed to MUC1 silenced EVs did not demonstrate an increase in c-Myc, cyclin E1 or D2 (Figure 18C) suggesting that MUC1 and c-Myc are the critical mediators of AML-EV induced MDSC proliferation.

We next sought to elucidate how MUC1 signalling promotes c-Myc expression in AML.

MUC1 signalling promotes c-Myc expression at a post-transcriptional level

To determine how MUC1 signalling promotes c-Myc expression, AML cells silenced for MUC1 expression were quantified for c-Myc mRNA by qPCR. Figure 19 shows that MUC1 silencing did not lead to any change in c-Myc mRNA, suggesting that MUC1 silencing down-regulates c-Myc protein expression at a post-transcriptional level.

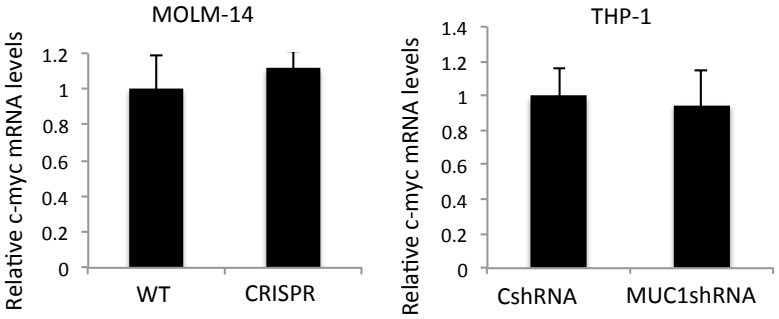


Fig 19. MUC1 silencing does not alter c-Myc mRNA levels. RNA was isolated from MUC1 silenced cells and subjected to qPCR with primers against c-Myc. C-Myc expression in MUC1 silenced MOLM-14 and THP-1 is quantified relative to control cells, summary of three experiments shown (n=3).

Post-transcriptional regulation of gene expression can be mediated through microRNA binding target genes with complementary sequences[497]. miR34a has previously been reported to be a key regulator of MDSC expansion and is a known regulator of c-Myc expression, binding to a complementary sequence in the 3'UTR of c-Myc mRNA[498]. We hypothesized that MUC1 might promote c-Myc expression by altering miR34a expression.

miR34a regulates c-Myc expression in AML

We sought to validate that miR34a regulates c-Myc expression in AML. Indeed, over-expression of miR34a using lentiviral transduction of mir34a mimic, led to a reduction in c-Myc expression in MOLM-14 and THP-1 (Figure 20A). Alternatively, we sought to evaluate if silencing miR34a could re-capitulate the c-Myc expression seen in MUC1 wildtype AML cells. MOLM-14 cells were over-expressed for miR34a, in order to obtain sufficient miR34a levels for silencing. Subsequently, miR34a was silenced in these cells using lentiviral transduction of miR34a-ZIP, demonstrating a dramatic increase in c-Myc

expression (Figure 20B). Furthermore, in MUC1 silenced THP-1 AML cells, previously shown to have low c-Myc expression (Figure 18B), silencing of miR34a demonstrated similar results, with significant increase in c-Myc levels (Figure 20C).

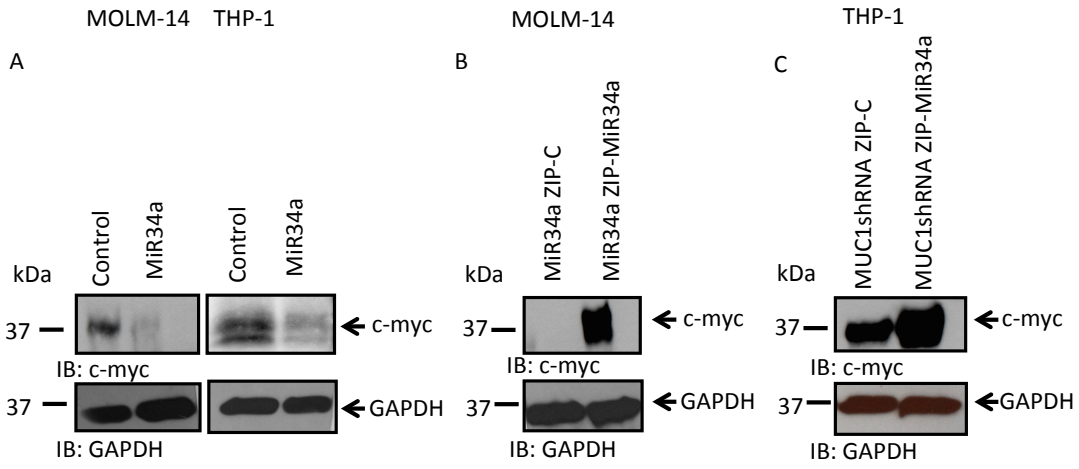


Figure 20. Transduction with miR34a mimic or ZIP-miR34a results in altered c-Myc expression in AML blasts. MUC1 regulates c-Myc expression via miR34a in AML cells. MOLM-14 and THP-1 cells were transduced with miR34a-mimic or control, using lentiviral transduction. **(A)** Lysates were prepared and c-Myc expression was assessed using western blot analysis. MOLM-14 AML cells over-expressing miR34a were then silenced for miR34a, by lentiviral transduction of miR34a-ZIP or control (n=2). **(B)** Lysates were prepared and c-Myc expression was assessed using western blot analysis. THP-1 AML cells silenced for MUC1 expression using specific MUC1 shRNA, were silenced for miR34a, using lentiviral transduction of miR34a-ZIP or control (n=3). **(C)** Lysates were prepared and c-Myc expression was assessed using western blot analysis (n=2).

miRNA34a levels are increased in MUC1 silenced AML cells and Extra-cellular Vesicles derived from these cells.

To determine if MUC1 expression effects the expression of the miRNA miR34a, MUC1 silenced and control MOLM-14 and THP-1 AML cells had their miRNA34a expression

quantified by q-PCR. Both MUC1 silenced AML cell lines had a 13-fold increase in miR34a expression (Figure 21 A and B). This finding was confirmed in Extra-cellular Vesicles isolated from MUC1 silenced AML cells (Figure 21 C and D).

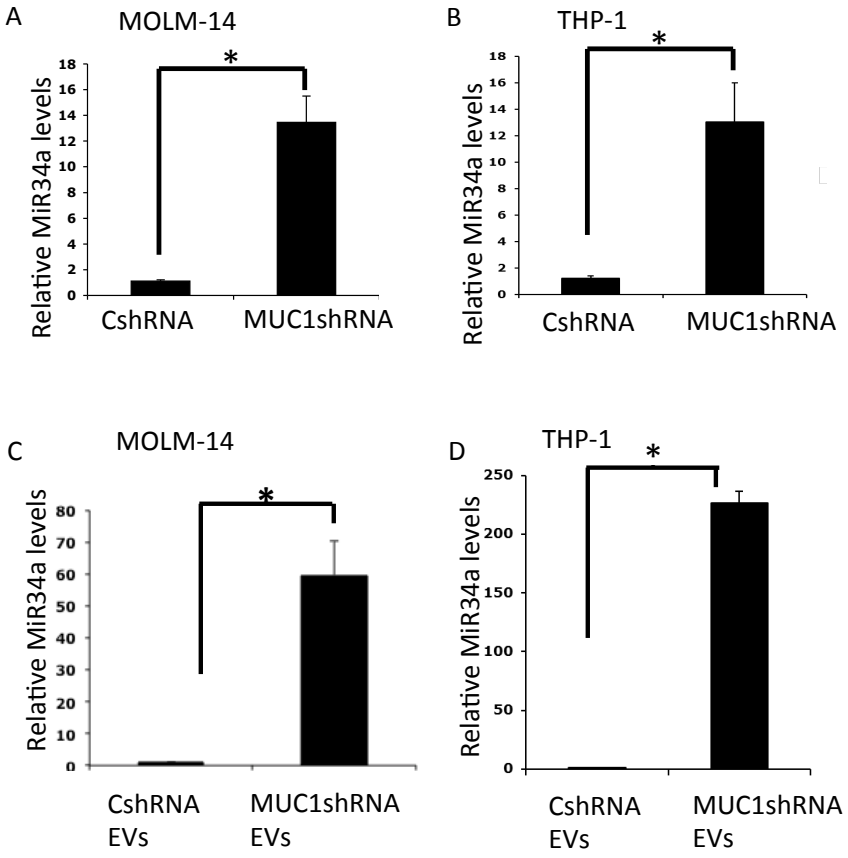


Figure 21. miRNA34a levels are increased in MUC1 silenced AML cells. Stably transduced cell lines silenced for the expression of MUC1 protein were generated by lentiviral transduction of shRNA or control vector. RNA was isolated from cells and subjected to qPCR with primers against miR34a. miR34a expression in MUC1 silenced MOLM-14 (A) and THP-1 (B) is quantified relative to control cells, summary of three experiments shown. Extracellular Vesicles were isolated from MUC1 silenced MOLM-14 (C) and THP-1 (D) cells as described. RNA was isolated from cells and subjected to qPCR with primers against miR34a. miR34a is quantified relative to control cells, summary of (n=3) experiments are shown.

Targeting miR34a in AML cells alters the expansion of MDSCs in co-cultured healthy donor PBMCs.

We next confirmed the critical role of miR34a in regulating MDSC expansion in AML. Overexpression of miR34a in wild type MOLM-14 and THP-1 cells by lentiviral transduction resulted in decreased capacity of the AML cells to induce expansion of MDSCs when co-cultured with normal PBMCs (Figure 22A). Furthermore, a co-culture of healthy PBMCs with MOLM-14 (Figure 22B) and THP-1 cells (Figure 22C) silenced for miR34a expression, resulted in a corresponding increase in MDSCs. Importantly, altering MUC1 or miR34a levels in MOLM-14 or THP-1 was not associated with changes in the rate of apoptosis, which might have otherwise accounted for this change in MDSC expansion (Figure 22D).

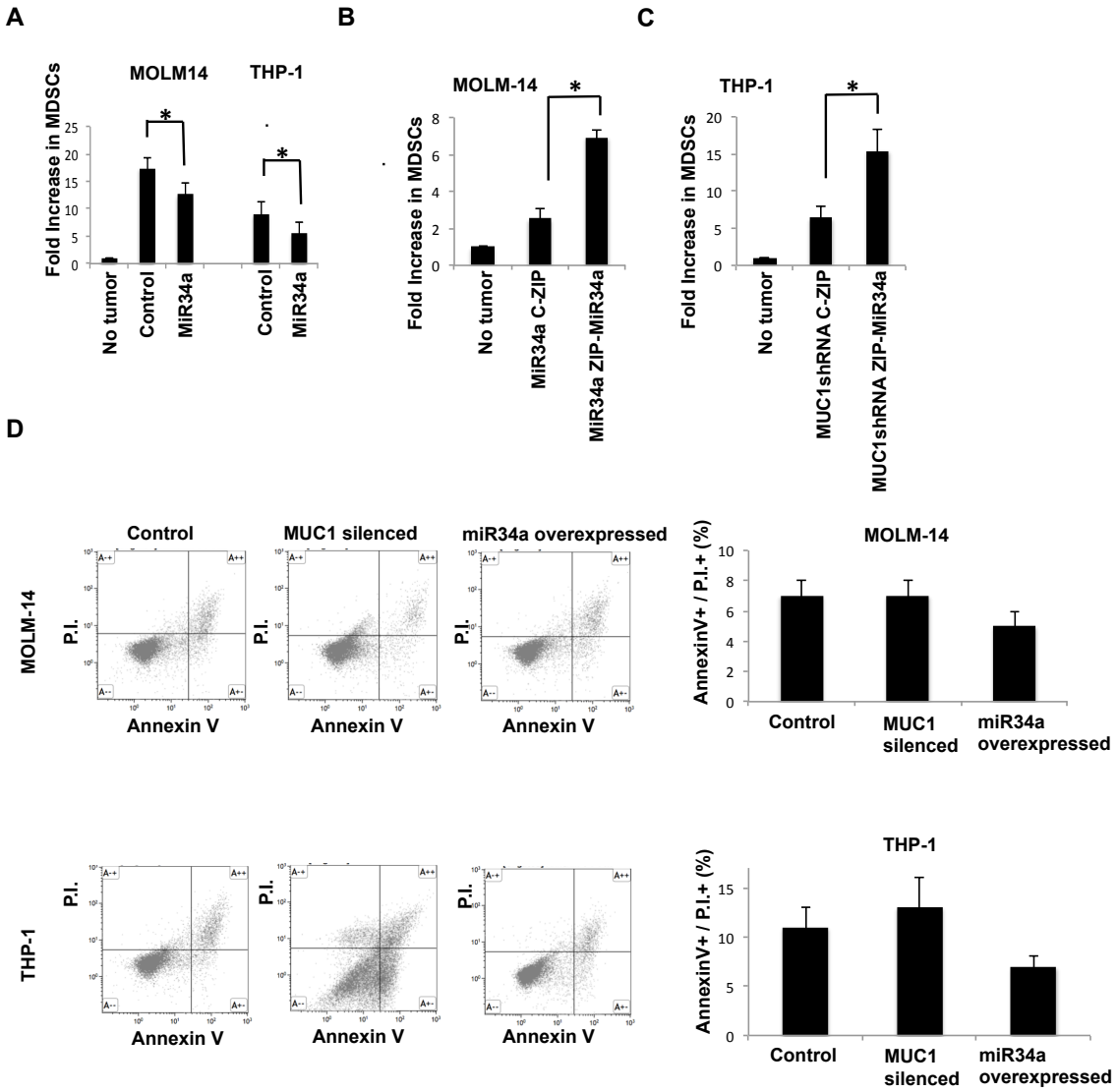


Figure 22. Targeting miR34a alters MDSC expansion in co-cultured PBMCs. Healthy PBMCs were co-cultured with irradiated, fluorescently labelled AML cells with over-expressed miR34a levels, for five days at a ratio of 100:1 (PBMC:AML). After co-culture, cells were analysed by Flow Cytometry and fluorescently labelled blast cells were excluded, CD11b+/HLADR-/CD33+ MDSCs were quantified as a percentage of immature CD11b+/HLADR- myeloid cells. **(A)** Summary of three independent experiments is shown for MOLM-14 and THP-1 ($p<0.05$). Similarly, MDSCs were detected in co-culture of PBMCs with miR34a silenced **(B)** MOLM-14 ($n=3$, $p<0.05$) and **(C)** THP-1 AML cells ($n=3$, $p<0.05$). **(D)** MOLM-14 and THP-1 were silenced for MUC1 expression or over-expressed

for miR34a, by lentiviral transduction, as described. Cells in log phase were stained for Annexin V and P.I. and analysed by Flow Cytometry. Summary of three independent experiments and representative plots are shown.

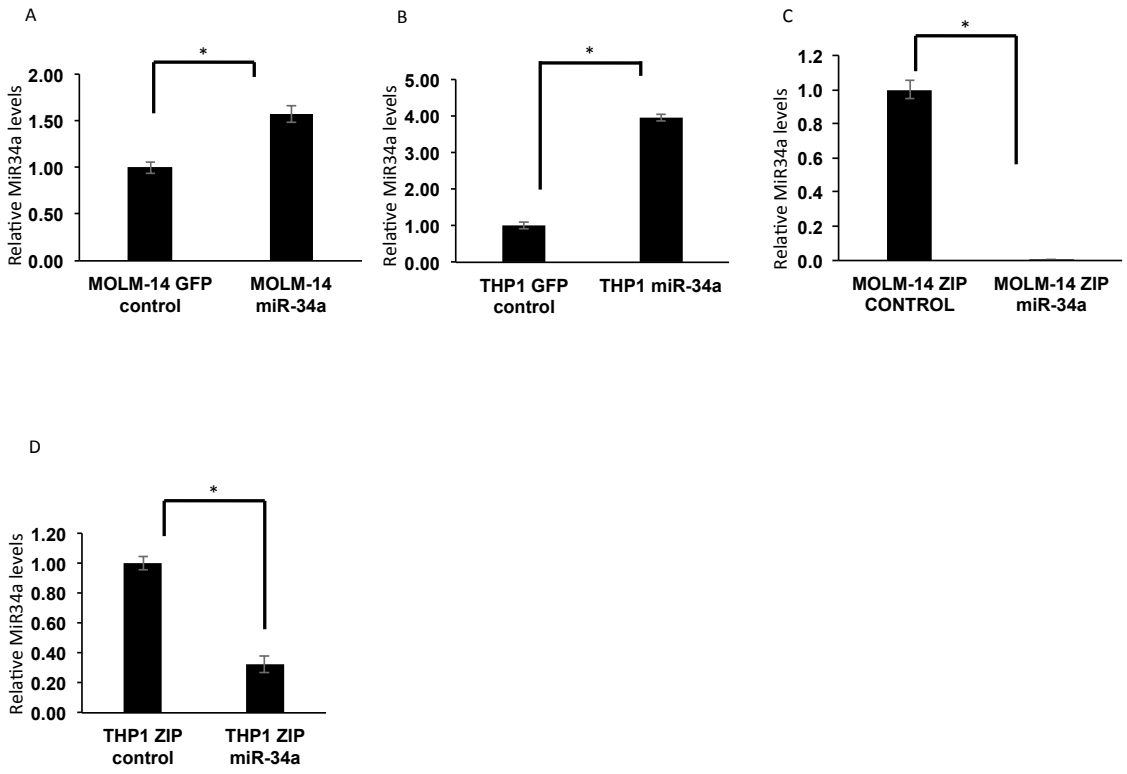


Figure 23. Transduction with miR34a mimic or ZIP-miR34a results altered miR34a in AML blasts. miR34a was stably overexpressed in MUC1 expressing wildtype (A) MOLM-14 and (B) THP-1 cells by lentiviral transduction of miR34a-mimic. RNA was isolated as before, and quantified for miR34a using qPCR (n=2,$p<0.05$). miR34a was stably silenced in (C) miR34a over-expressing MOLM-14 and (D) MUC1 silenced THP-1 cells by lentiviral transduction of miR34a-ZIP. RNA was isolated as before, and quantified for miR34a using qPCR (n=2,$p<0.05$).

MUC1 silencing increases miR34a levels, but not precursor pre-MIR34a

We had so far elucidated that MUC1 induces the expansion of MDSCs via the suppression of miR34a, which drives the promotion of c-Myc expression, which is exported by EVs to MDSCs causing their proliferation.

It was unclear as to how MUC1 signalling could negatively regulate the expression of a MicroRNA. miRNAs are first transcribed as large RNA precursors called *pri-miRNAs* which are processed in the nucleus by the RNAse III enzymes Drosha and PASHA into *pre-miRNAs* of roughly 70-nucleotides in length. The pre-miRNAs are exported into the cytoplasm and undergo processing by the RNAse III enzyme Dicer generating the final mature miRNA, which consists of double-stranded RNA of roughly 22 nucleotides in length[34,35].

Following this processing, DICER bound mature miRNA forms a complex known as the RNA-induced silencing complex (RISC), made up of DICER, miRNA, a transport protein called transactivating response RNA-binding protein (TRBP) and Argonaut2 (Ago2). TRBP recruits Ago-2 to the RISC, whereby it serves to cleave the target mRNA strand complementary to their bound miRNA[36].

In order to elucidate if MUC1 affects the transcription of the precursor forms of miR34a or the processing of the pre-miRNA to the mature version, we performed q-PCR on MUC1 silenced AML cells, and their respective controls, for both the mature miR34a and the precursor pre-miR34a transcripts.

Figure 24 demonstrates that when MUC1 is silenced, miR34a levels increase as previously seen. However the levels of pre-miR34a stay the same, indicating that the negative regulation that MUC1 has of miR34a expression happens at the post-transcriptional stage.

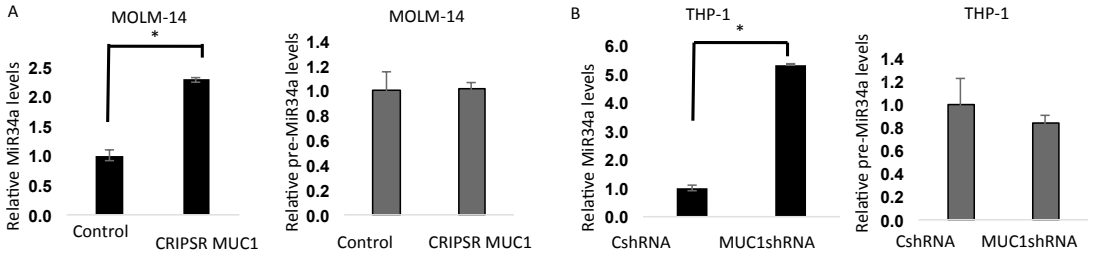


Figure 24. MUC1 silencing increases miR34a levels, but not of precursor pre-miR34a. q-PCR for miR34a and pre-miR34a transcripts was performed on MUC1 silenced AML cell lines MOLM-14 **(A)** and THP-1 **(B)**. Representative q-PCR is shown of three experiments.

MUC1 silencing increases the expression of the miRNA processing protein DICER1

Our data suggested that MUC1s negative regulation of miR34a expression happens at the post-transcriptional stage. The DICER protein processes pre-miRNAs into mature miRNAs so we sought to elucidate the effect of MUC1 signalling on the expression of DICER. Westerns blots for DICER were performed on lysates from AML cells silenced for MUC1 expression (Figure 25A) and AML cells treated with the MUC1 inhibitor GO-203, or the control compound CP3 (Figure 25B). These immune-blots demonstrate that MUC1 silencing or inhibition results in a dramatic up-regulation of DICER protein and mRNA expression. In parallel, there was no change in the levels of Argonaut-2 protein, which functions after the processing of pre-miRNA to mature miRNA (Figure 25 A and B).

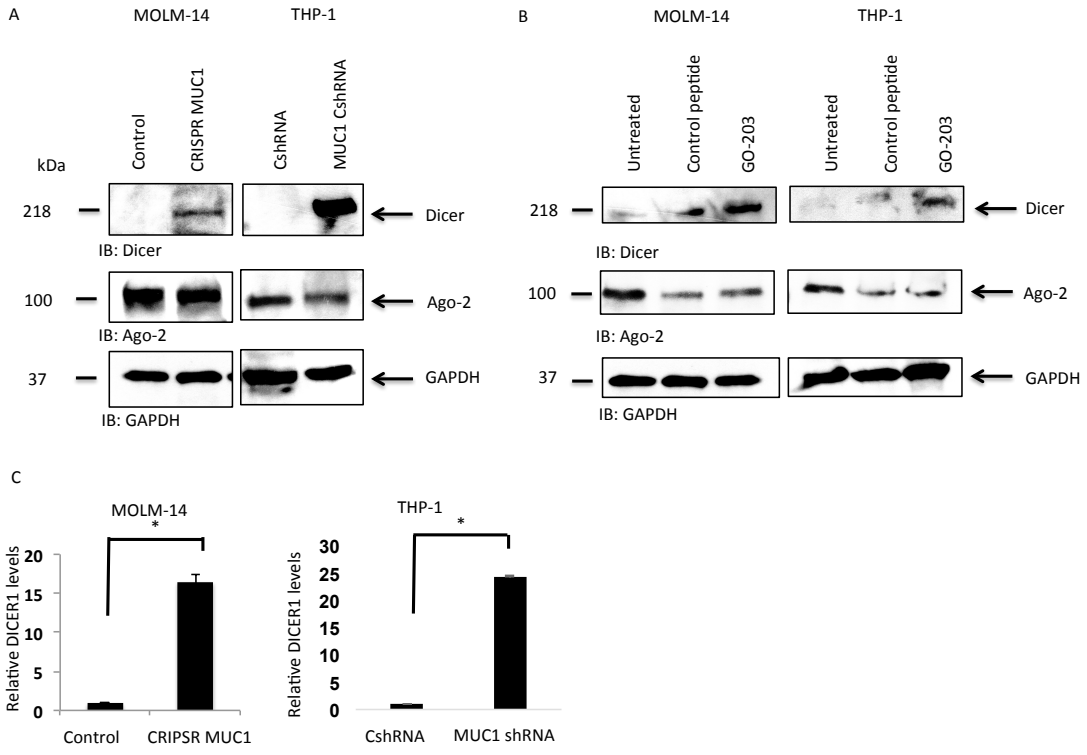


Figure 25. MUC1 silencing and inhibition results in the up-regulation of DICER protein. MUC1 was targeted by silencing **(A)** and inhibition **(B)** and lysates were immune-blotted for the expression of DICER and Argonaut-2 proteins (n=2). MUC1 inhibition was achieved by the daily treatment of AML cells with the sub-lethal dose of 1uM GO-203, or control compound, for three days. RNA isolated from MUC1 silenced cells was subjected to q-PCR for DICER1 mRNA in MOLM-14 and THP1 (n=2) **(C).**

MUC1 down-regulates DICER expression by repression of the transcription factor c-jun

We next sought to elucidate how MUC1 signalling down-regulates DICER expression in AML cells. The increase in DICER mRNA upon MUC1 silencing (Figure 25C) suggested a transcription factor may be mediating this pathway. Using transcription binding site prediction software (Appendix E), the AP-1 transcription factor family member cJUN was predicted to most strongly regulate DICER transcription. Immunoblots of MUC1 silenced

AML cell lines showed increased total and phosphorylated (activated) cJUN in MUC1 silenced cells compared to control (Figure 26A). To determine if MUC1 regulated c-jun at the transcription level, q-PCR for cJUN was performed showing dramatically increased levels of cJUN upon MUC1 silencing (Figure 26 C). Similarly an mRNA array of AML blasts from patient samples treated with control or the MUC1 inhibitor (GO-203), showed an increase in cJUN mRNA (p=0.007) (Figure 26B). Finally, to confirm cJUN as the critical regulator of DICER protein expression in AML, MUC1 silenced AML cells were treated with increasing doses of a cJUN inhibitor, demonstrating a dose dependent decrease in DICER expression (Figure 26D).

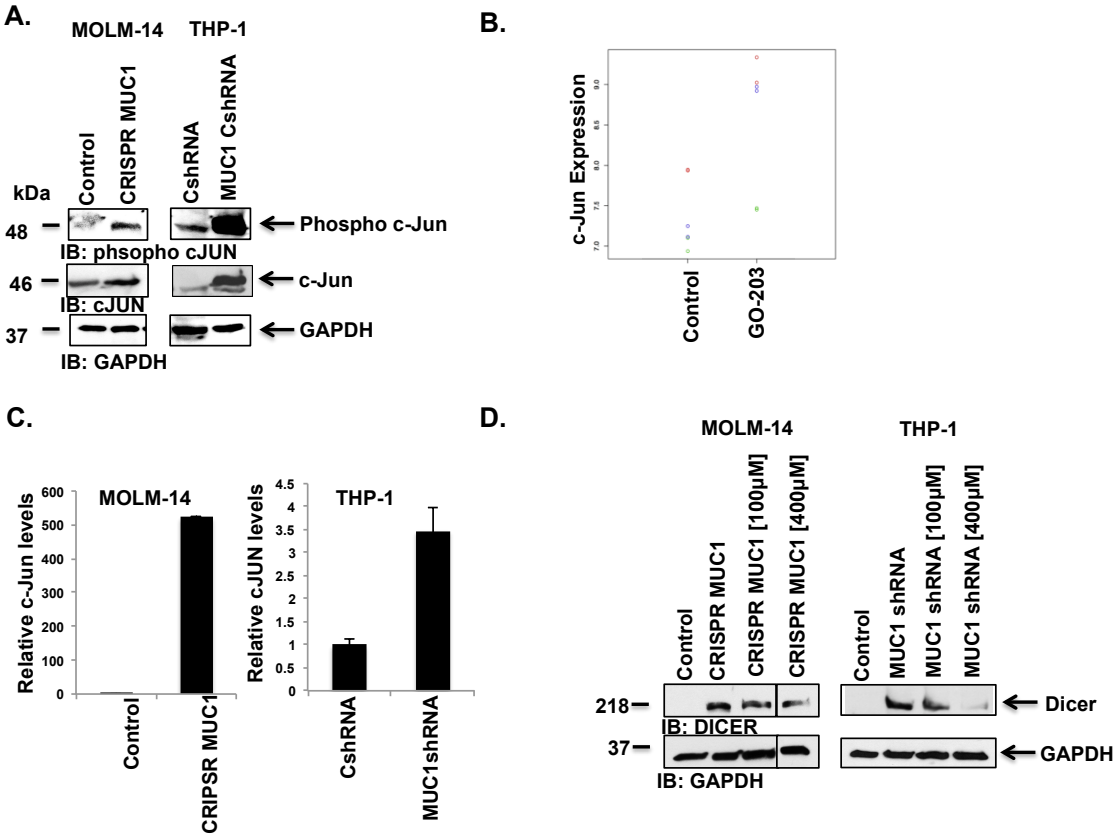


Figure 26. MUC1 signalling leads to decreased DICER expression by suppressing cJUN transcription and activity. MUC1 was silenced by lentiviral transduction of shRNA in

THP-1 cells and CRISPR/Cas9 in MOLM-14 cells. Immunoblotting of lysates was performed for phosphorylated cJUN, total cJUN and GAPDH **(A).** MRNA array was performed on three GO-203 or control treated primary AML samples. A plot showing the relative expression of cJUN mRNA is shown (p=0.07) **(B).** RNA was isolated from MUC1 silenced AML cell lines and qPCR was performed to quantitate cJUN transcripts **(C)** (n=2)**.** MUC1 silenced AML cells were treated for 2 hours with 0uM, 100uM or 400uM of cJUN inhibitor, lysates were immunoblotted for DICER and GAPDH **(D)** (n=2).

MUC1 inhibition leads to increase in the majority of mRNAs in AML cells. We next performed an array to identify other microRNAs that were similarly impacted by silencing with MUC1. MicroRNA array of MUC1 silenced MOLM-14 and THP-1 cells demonstrated a profound global up-regulation of the vast majority of microRNAs (Fig 27), consistent with the increase in DICER expression we had previously observed. Of the panel of 801 miRNAs arrayed, MUC1 silenced MOLM-14 cells showed an increase in 786/801 (98.1%), of which 340 (42.4%) reached Bonferroni-corrected significance. Concordantly, MUC1 silenced THP-1 cells showed an increase in 698/801 (87.1%) miRNAs, of which 154 (19.2%) reached Bonferroni-corrected significance.

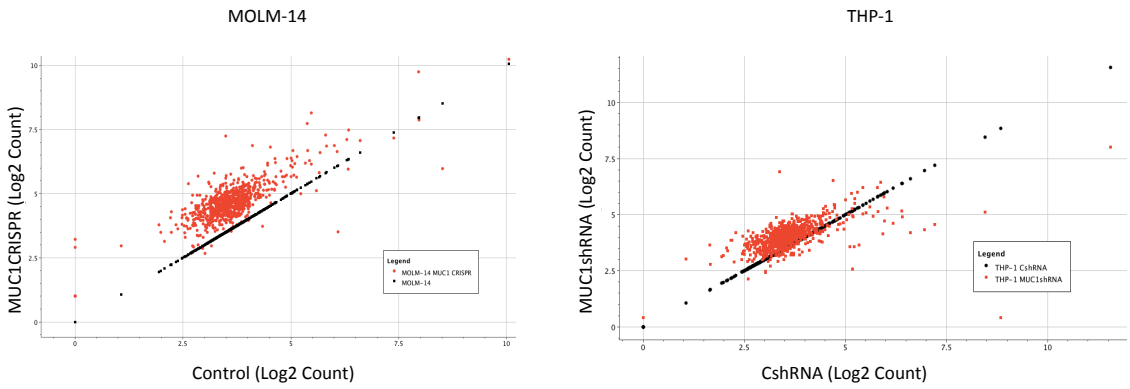


Figure 27. Silencing of MUC1 in AML cells increases the global expression of miRNAs MicroRNA array of MUC1 silenced MOLM-14 and THP-1 cells was performed and

illustrated by scatter plots showing Log2 abundance of 801 profiled miRNAs. Summary of three experiments is shown.

In the present study we have elucidated that MUC1 signalling in AML, drives MDSC accumulation by promoting c-Myc expression in EVs. MUC1 signalling promotes c-Myc expression by repressing miR34a production, via the suppression of DICER transcription, mediated by the repression of cJUN transcription.

While the full elucidation of the mechanism by which MUC1 may suppress cJUN transcription is beyond the scope of these studies, it has been recently shown that MUC1 has hyper-methylating properties[499], which may globally suppress gene transcription. Treatment with the MUC1 inhibitor GO-203, lead to de-methylation and increased expression of several critical genes in a model of AML (Kufe et al unpublished data). We therefore sought to elucidate if hypomethylation of AML cells, using the hypomethylating agent (HMA) Decitabine, would lead to increased transcription of cJUN and DICER, mimicking the effects of MUC1 inhibition.

Treatment with a hypomethylation agent increases phospho- and total cJUN and DICER, with a corresponding decrease in c-Myc expression.

THP-1 and MOLM-14 AML cells were treated with a sub-lethal dose of the HMA Decitabine, (for dose curves see Appendix D), daily for three days and cells were harvested at day 7. Day 7 was chosen as previous studies have suggested that peak demethylation occurs at this time point[500]. Immuno-blots for cJUN and DICER demonstrate an increase in expression with 5nm of Decitabine, which is not further increased by dose increment, suggesting a plateau effect (Figure 28). Concordantly, c-Myc expression is reduced in HMA treated AML cells, in keeping with described reports in lymphoma[501], and mimicking the effect seen with MUC1 silencing.

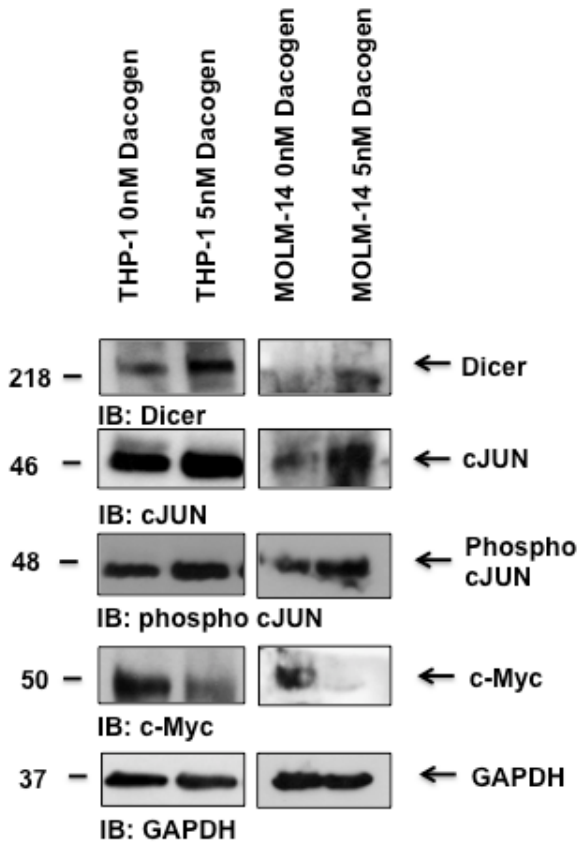


Figure 28. Treatment of AML cells with Decitabine increases the expression of cJUN and DICER, with a corresponding reduction in c-Myc expression (n=2).

4.5. Discussion

MUC1 is a critically important oncoprotein in AML, whose signalling limits the immune response[493,502]. We had observed an apparent requirement for cell-cell contact in the process of MDSC expansion and therefore hypothesized that MUC1, a protein expressed both on the surface of the tumour cell and also in secreted EVs, might be involved in this process.

To test this hypothesis, we generated AML cell lines silenced for the expression of MUC1-C, the active cytoplasmic domain of the MUC1 molecule[30]. Thereafter, we co-

cultured MUC1 silenced AML cells with healthy donor PBMCs, demonstrating a significant abrogation in MDSC expansion. To support the argument that MUC1 was involved in soluble factor independent mediated expansion of MDSCs, we performed cytokine arrays on MUC1 silenced and control AML cells, demonstrating no significant difference in any secreted cytokine, notably GM-CSF, IL-1B and Il-6, implicated in MDSC expansion.

We therefore sought to elucidate a soluble factor independent mechanism by which MUC1 might be inducing the expansion of MDSCs. Given the observed importance of Extra-cellular vesicle (EV) export in the process of MDSC expansion (see Chapter 3), we probed AML-EVs for the presence of MUC1 and c-Myc. C-Myc is of known importance in leukaemogenesis, conferring therapeutic resistance[37] and increased cell proliferation via its downstream targets the pro-proliferative cyclins D2 and E1. Moreover, MUC1 has been demonstrated to drive c-Myc expression in myeloma and lung cancer in a transcription factor dependent mechanism[38,39]. We subsequently demonstrated that AML EVs contain MUC1 and c-Myc and that MUC1 silencing results in down-regulation of c-Myc in parental AML cells and EVs. Furthermore, c-Myc containing EVs lead to an increase in c-Myc expression and the pro-proliferative cyclins D2 and E1 in EV exposed MDSCs. Crucially, MDSC exposed to EVs derived from MUC1 silenced AML cells did not show an increase in c-Myc or downstream cyclins.

We then sought to determine how MUC1 promotes c-Myc signalling in AML. As MUC1 silencing did not result in altered levels of c-Myc mRNA, we looked for miRNAs which might mediate a post-transcriptional mechanism of c-Myc regulation. Using prediction software, we identified miR34a as one of several likely regulators of c-Myc expression. Interestingly, miR34a, a target of p53, has been shown to be crucially involved in regulating the expansion of MDSCs[24]. We first validated miR34a as a regulator of c-Myc expression by over-expressing and silencing miR34a in AML cells, demonstrating decreasing and increasing protein expression of c-Myc, respectively. We subsequently investigated the effect of MUC1 signalling on miR34a expression. We found that MUC1

silencing resulted in a significant upregulation of tumour miR34a expression, and moreover, a dramatic upregulation of upwards of 200 fold increase in miR34a expression in AML derived EVs. This discordance between tumour and EV miR34a suggests that miR34a may be preferentially exported into EVs. The reasoning for this remains unclear. As miR34a is a tumour suppressor, MUC1 silenced tumour cells may be preferentially exporting miR34a as a means of disposing of this anti-tumour miRNA. Concordantly, MUC1 expressing wildtype AML cells may preferentially down regulate miR34a export, as a means of de-regulating MDSC expansion.

To confirm that miR34a was a critically important regulator of MDSC expansion in AML, we over-expressed miR34a in wildtype AML cells, demonstrating that MDSC expansion was significantly abrogated. Subsequently miR34a expression was silenced, firstly in MUC1 silenced THP1 cells, and subsequently in miR34a overexpressing MOLM14 cells. miR34a silenced AML cells elicited a significant increase in MDSC expansion. In this study we have demonstrated that MUC1 regulates c-Myc expression in AML via a post-transcriptional, miR34a dependent mechanism.

We next investigated how MUC1 signalling negatively regulates the expression of miR34a. In order to elucidate if MUC1 affects the transcription of the precursor forms of miR34a or the processing of the pre-miRNA to the mature version, we performed q-PCR on MUC1 silenced AML cells, and their respective controls, for both the mature miR34a and the precursor pre-miR34a transcripts. We demonstrated that MUC1 silencing increases miR34a levels, but not of precursor pre-miR34a, suggesting that MUC1s negative regulation of miR34a expression happens at the post-transcriptional stage. The DICER protein processes pre-miRNAs into mature functional miRNAs so we sought to elucidate the effect of MUC1 signalling on the expression of DICER. AML cells silenced for MUC1 expression and AML cells treated with the MUC1 inhibitor GO-203, or the control compound CP3, were immune-blotted for the DICER protein. The resultant immunoblots demonstrate that MUC1 silencing or inhibition results in a significant up-regulation of DICER expression. Notably, there was no change in the levels of the

Argonaut-2 protein, which functions after the processing of pre-miRNA to mature miRNA.

To determine if MUC1 regulates DICER at the pre- or post-transcriptional level, q-PCR for DICER1 mRNA was performed on MUC1 silenced or inhibited cells, demonstrating a concurrent significant increase in DICER mRNA. We therefore sought to elucidate how MUC1 suppresses DICER transcription. Using prediction software, we identified cJUN as a likely promoter of DICER transcription. We then confirmed that MUC1 silencing leads to an increase in the mRNA and protein levels of cJUN, as measured by qPCR and immune-blotting, respectively. In support of this observation, an mRNA array of MUC1 inhibitor treated primary patient blasts demonstrated a significant increase in cJUN mRNA, when compared to control peptide treated cells. Furthermore, treatment of MUC1 silenced AML cells with a peptide inhibitor of cJUN lead to a dose dependent reduction in DICER expression, confirming cJUN as a key promoter of DICER expression.

As there is emerging evidence that MUC1 may repress gene transcription by acting as a hyper-methylator[503,504], we finally sought to elucidate if treatment with a hypomethylating agent could restore cJUN expression, and subsequently, DICER expression. Treatment with sub-lethal doses of a hypomethylating agent, decitabine, lead to an increase in expression of cJUN and DICER. Concordantly, c-Myc expression is reduced in HMA treated AML cells, mimicking the effect seen with MUC1 silencing.

In the next chapter, we detail our efforts to exploit the elucidation of this signalling pathway, to target the expansion of immune suppressive MDSCs, *in vitro* and *in vivo.*

Chapter 5. MDSCs can be targeted by MUC1 inhibition, or by the use of a novel hypomethylation agent SGI-110.

5.1. Introduction

In the previous chapter, we have demonstrated that AML MUC1 signalling leads to a proliferative expansion of MDSCs via the export of c-Myc in AML EVs to MDSCs. MUC1 signalling promotes c-Myc expression in AML cells and AML EVs, and this is mediated by the down-regulation of the microRNA miR34a. MUC1 signalling down-regulates miR34a expression via the suppression of the miRNA processing protein, DICER. MUC1 signalling represses DICER expression by down-regulating expression of the AP-1 family transcription factor cJUN, which normally drives DICER transcription.

The elucidation of this important signalling pathway offers several possibilities for targeting immune-suppressive MDSCs. MUC1 inhibition may most potently target MDSCs, as it is the most upstream in this pathway, but its effects may be more wide spread and not just specific to MDSC targeting. Similarly, restoring cJUN expression via the delivery of a recombinant protein offers another possibility, although would also have many other off-target effects as cJUN can act as a proto-oncogene[505] causing pro-tumourigenic effects. Restoring DICER expression may increase anti-tumour miRNAs such as miR34a, thereby targeting MDSCs, but the DICER protein is very large (>220kDA), rending delivery into cells functionally challenging. The direct restoration of miR34a expression, via the delivery of recombinant miR34a, offers a more targeted approach to reduce MDSC expansion, although this will also have anti-proliferative effects on AML cells, as described in Chapter 4.

We decided to first attempt targeting MDSCs using MUC1 inhibition, with the acceptance that the effects of MUC1 inhibition are many, and some will be not specific to MDSC expansion. While high doses of the MUC1 inhibitor GO-203 are toxic to AML cells (See dose curves in Appendix D), sub-lethal treatment with 1uM increased DICER expression (Chapter 4) and we hypothesized may therefore reduce MDSC expansion at least in part by a miR34a mediated effect on c-Myc export.

Our previous work exploring the regulation of DICER expression (See Chapter 4), demonstrated that DICER expression was both transcriptionally regulated, by the transcription factor cJUN, and also epigenetically regulated, as treatment with sub-lethal doses of the hypomethylating agent decitabine, was shown to increase, both cJUN and DICER expression in AML cells. This is in keeping with published work demonstrating that Histone deacetylase Inhibition increased DICER expression in T cell leukaemia[506]. The caveat to these statements is that hypomethylation may only indirectly increase DICER expression, solely via its effect on cJUN expression. There is emerging evidence that MUC1 may be involved in the hyper-methylation of cancer cells[503,504], raising the possibility that MUC1 regulates the expression of DICER by both transcriptional and post-transcriptional mechanisms.

We therefore hypothesized that treatment with hypomethylating agents might also target MDSCs, as part of their panel of immune modulatory effects. Supporting evidence for this hypothesis, is the observation that melanoma bearing mice treated with decitabine, a first generation hypomethylating agent, bore fewer MDSCs[507].

Using decitabine to target MDSCs poses an attractive option, as decitabine is already a standard of care drug for patients with relapsed/refractory AML, or those who are not fit enough for conventional cytotoxic therapy. However, the half-life of decitabine is extremely short at 15-25 minutes[508], due to rapid inactivation by liver cytidine deaminase, and the dosing schedule in humans ranges from every 8 hours, to once daily infusions. Second-generation DNMT inhibitors have been developed such as the drug SGI-110, designed to enhance the efficacy of decitabine by combining it with deoxyguanosine. This confers resistance to degradation by cytidine deaminase, therefore increasing the half-life of the drug. A phase II trial of SGI-110 in relapsed and refractory or elderly newly diagnosed AML patients demonstrated an overall remission rate of 16% in the control group and 42.5% in the SGI-110 group[63]. Given the improved pharmacokinetics of SGI-110 over decitabine, we decided to elucidate the effect of hypomethylation on the expansion of MDSCs, using this novel compound.

5.2. Aims

In the present study we sought to target MDSCs using MUC1 inhibition *in vitro* and in our *in vivo* murine model of AML. In parallel, we tested the effect of a novel hypomethylating agent, SGI-110, on the expansion of MDSCs *in vitro* and *in vivo*.

5.3. Materials and Methods

Immuno-competent murine model of AML: GO-203

To achieve MUC1 inhibition, we used the cell-penetrating peptide inhibitor (GO-203) which blocks the MUC1-C CQC motif and inhibit MUC1-C homodimerization[131]. To achieve a longer half-life in *in vivo* settings, GO-203 was obtained pre-packaged in a nano-particle formulation.

On day 1, $1x10^5$ GFP tagged C1498 (TIB-49) (C57BL/6 syngeneic) murine AML cells were injected retro-orbitally into C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). Simultaneously, mice to be treated with GO-203 received one intra-peritoneal dose of 15mg/kg of GO-203 nano-particles. At day 14, all mice were euthanized. Bone marrow cells were flushed from the femur bone marrow cavity into sterile RPMI 1640 media. Spleens were emulsified to obtain a cell suspension. BM and spleen cells were washed prior to further analysis by flow cytometry. Engraftment, as detected by GFP-positive cells in the BM, was assessed by flow cytometry. BM and spleens were analysed by FACS for MDSC quantification using the markers CD11b and Gr1.

Immuno-competent murine model of AML: SGI-110

On day 1, $1x10^5$ GFP tagged C1498 (TIB-49) (C57BL/6 syngeneic) murine AML cells were injected retro-orbitally into C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). At day 12, mice began to show signs of establishing AML, such as reduced physical activity or visible orbital tumour (chloroma) development. Mice commenced daily subcutaneous treatments, for three days, of 1mg/kg SGI-110 or just the diluent as a control treatment. Cohorts of mice were euthanized at day 12 (before commencing treatment), day 16

(one day after finishing treatment) and day 19 (four days after finishing treatment). BM cells were flushed from the femur bone marrow cavity into sterile RPMI 1640 media. Spleens were emulsified to obtain a cell suspension. BM and spleen cells were washed prior to further analysis by flow cytometry. Engraftment, as detected by GFP-positive cells in the bone marrow, was assessed by flow cytometry. BM and spleens were FACS analysed for MDSC quantification using the markers CD11b and Gr1.

5.4. Results

MUC1 inhibition using a cell penetrating peptide GO-203, inhibits the expansion of MDSCs in vitro and in vivo

MOLM-14 and THP-1 AML cells were treated with a sub-lethal dose of the MUC1 inhibitor GO-203 or control peptide CP3 and co-cultured with healthy donor PBMCs. Treating AML cells with GO-203 lead to a significantly reduced expansion of MDSCs compared to control (Figure 29 A). Subsequently, immune-competent mice were injected with murine AML cells TIB-49 and simultaneously either a single dose of GO-203, or PBS. At day 14, all mice were euthanized and their femurs were assessed by flow cytometry for bone marrow engraftment and MDSC burden. Figure 29 B shows that mice treated with GO-203 had a reduction in bone marrow engraftment (Figure 29 B) although this was not significant. Mice treated with GO-203 had a significantly reduced MDSC burden ($p=0.01$) (Figure 29 C).

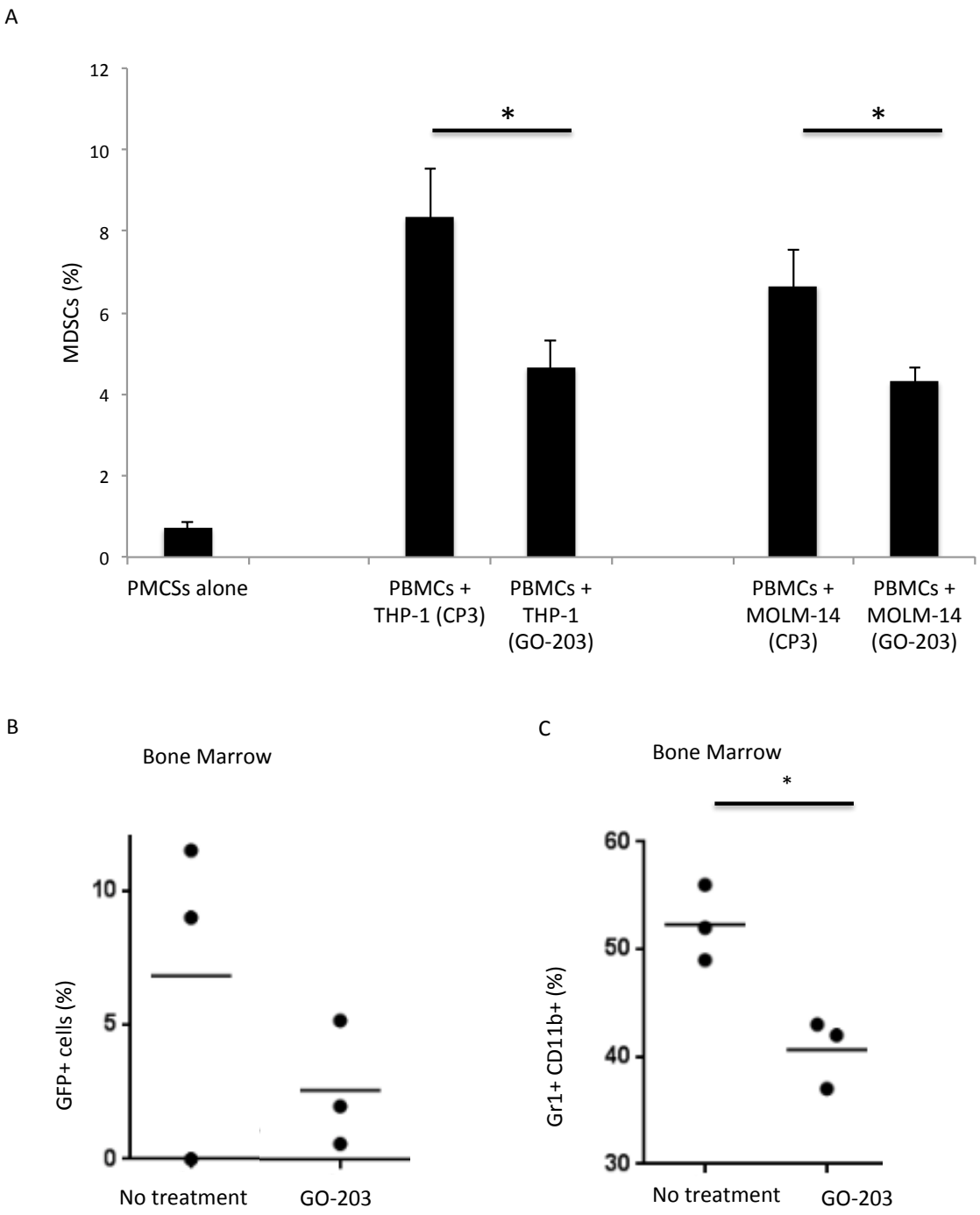


Figure 29. MUC1 inhibition inhibits the expansion of MDSCs *in vitro* and *in vivo*

MOLM-14 and THP-1 AML cells were treated with a sub-lethal dose of GO-203, 1um, once daily for three days, based on dose curves (Appendix 1). After three days, cells were washed, irradiated, fluorescently tagged and co-cultured with healthy donor

PBMCs, as previously described. After five days, MDSCs in the PBMC fraction were quantified by flow cytometry, demonstrating that the treatment of AML cells with GO-203 inhibits the expansion of MDSCs **(A)** (n=3). C57BL/6 mice were retro-orbitally injected with $1x10^5$ GFP tagged C1498 (TIB-49) cells and concurrently given one subcutaneous dose of GO-203, or PBS. At day 14, all mice were euthanized and their femurs were assessed by flow cytometry for BM engraftment as determined by GFP positive cells **(B)** and MDSC burden, as determined by Gr1+ CD11b+ cells **(C).**

A novel hypomethylating agent SGI-110, inhibits the expansion of MDSCs in vitro and in vivo

THP-1 and MOLM-14 cells were treated with SGI-110 or control (diluent) and co-cultured with healthy donor PBMCs. Treating AML cells with SGI-110 lead to a statistically significant reduction in expansion of MDSCs compared to control (Figure 30).

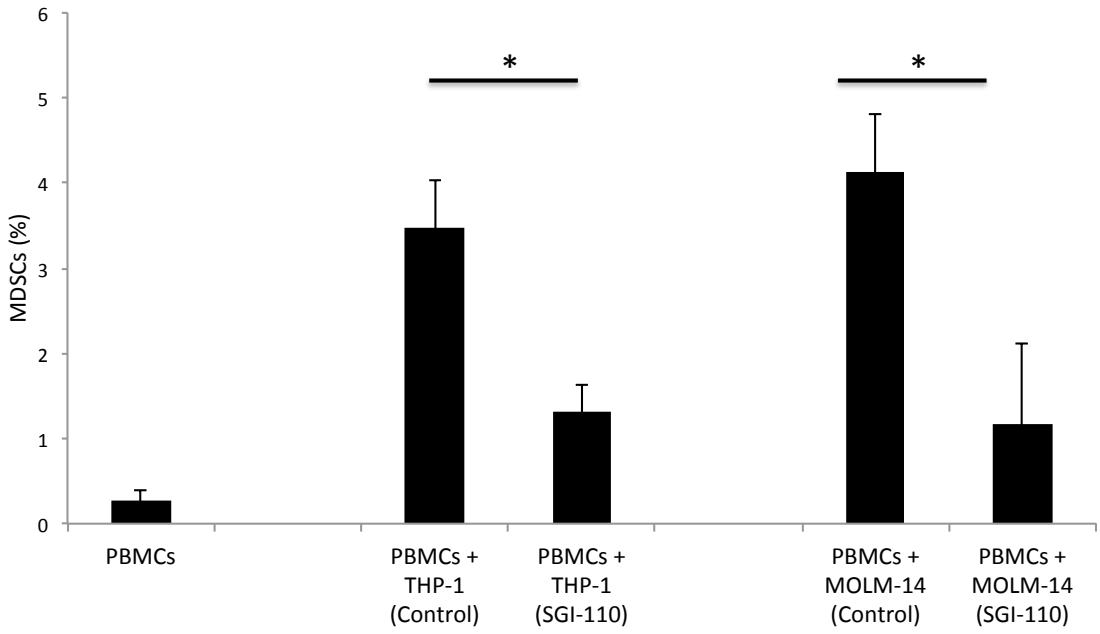
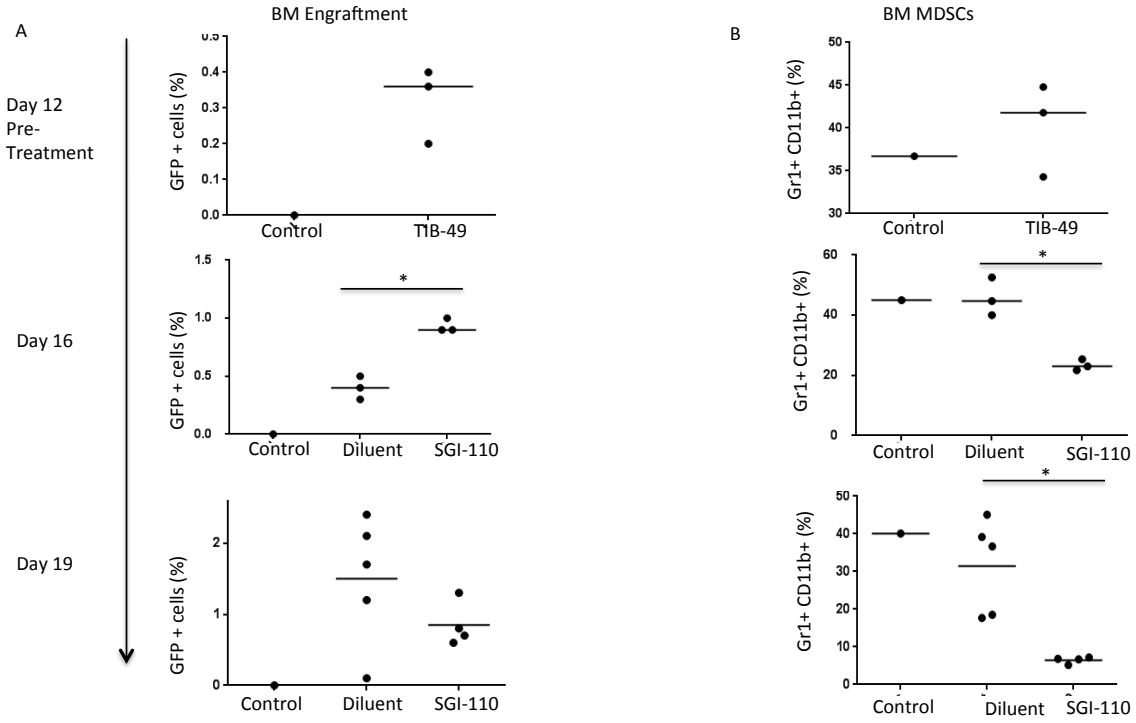


Figure 30. **The hypomethylating agent SGI-110 inhibits the expansion of MDSCs *in vitro.*** MOLM-14 and THP-1 AML cells were treated with a sub-lethal dose of SGI-110, 1um, twice daily for 48 hours, based on dose curves (Appendix 1). After a two day wash

out period, cells were washed, irradiated, fluorescently tagged and co-cultured with healthy donor PBMCs, as previously described. After five days, MDSCs in the PBMC fraction were quantified by flow cytometry (n=3).

Subsequently, mice were engrafted with murine AML and when they started to show signs of disease establishment, at day 12, commenced daily treatment with SGI-110 for three days. Cohorts of mice were euthanized at days 12, 16 and 24, and their BMs and spleens assessed for engraftment and MDSCs. Treatment with SGI-110 did not reduce engraftment in the BM or Spleen at days 16 or 24 days (Figure 31 A and C). Treatment with SGI-110 significantly reduced MDSCs at days 16 and 24 in both the BM (31 B) and the Spleens (31 D) of treated animals.



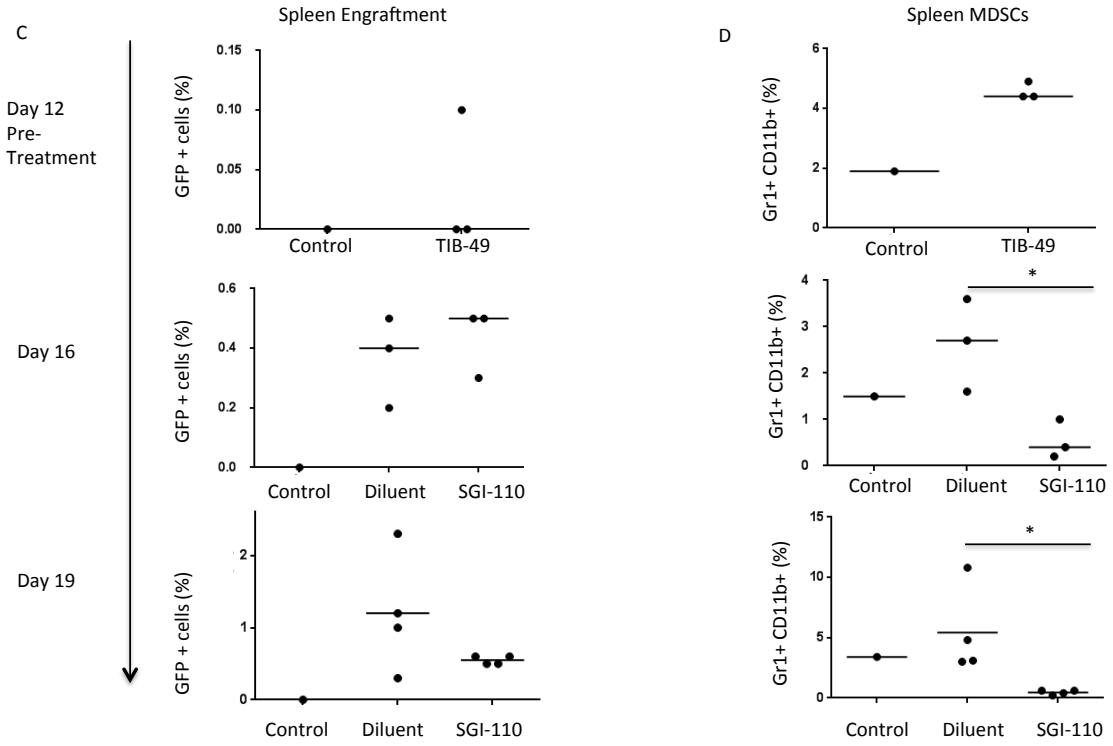


Figure 31. **The hypomethylating agent SGI-110 inhibits the expansion of MDSCs *in vivo*.** C57BL/6 mice were retro-orbitally injected with $1x10^5$ GFP tagged C1498 (TIB-49) murine AML cells. At day 12, mice commenced daily subcutaneous dosing of SGI-110, at 1mg/kg SGI-110 or diluent as a control treatment for 3 days. Cohorts of mice were euthanized at day 12 (before commencing treatment), day 16 (one day after finishing treatment) and day 19 (four days after finishing treatment). Bone marrow and spleen cells were analysed by flow cytometry. Engraftment was detected by GFP-positive cells in the bone marrow (A) and spleen (C). MDSCs were quantified in bone marrow (B) and spleen (D) using the markers CD11b and Gr1.

5.5. Discussion

In the present study we have demonstrated that MUC1 inhibition using a novel cell penetrating peptide GO-203, blunts the AML induced expansion of MDSCs *in vitro*. In the *in vivo* murine model of AML, treatment of mice with GO-203 lead to a significant reduction in MDSCs. This reduction of MDSCs cannot be fully attributed to a direct effect on MDSCs, as the small (and non-significant) reduction in tumour burden seen, may also lead to a reduction in expansion in MDSCs. While this limits our interpretation of this experiment, it offers up an exciting translational opportunity, to target both the tumour cell and surrounding immune suppressive stromal milieu.

We subsequently demonstrated that hypomethylation using a novel hypomethylating agent SGI-110, significantly reduces the AML induced expansion of MDSCs *in vitro and in vivo*. Indeed in both *in vitro and in vivo* models, the reduction in MDSC burden was more dramatic than in the studies using MUC1 inhibition. Furthermore, in the *in vivo* model of murine AML, MDSC depletion occurred by day 16, several days before tumour burden began to fall. This suggests a more direct effect of hypomethylation on MDSCs, independent of tumour burden, and might lead us to hypothesize that the trend towards a reduction in tumour burden in SGI-110 treated animals seen at day 19, might be in part mediated by a reduction in immune suppressive MDSCs, perhaps leading to greater immune cell mediated anti-tumour cytotoxicity.

Chapter 6. Discussion

Tumour cells promote an immunosuppressive milieu that inhibits intrinsic immune effectors and promotes the growth of disease[509]. Immature myeloid cells such as Myeloid-derived Suppressor Cells (MDSCs) have potent immune supressing activity and play a critical role in creating the immunosuppressive milieu of the tumour microenvironment[30]. MDSCs modulate the interactions between immune effector cells and malignant cells resulting in tumour progression[510], poor outcomes[266], and decreased effectiveness of immunotherapeutic strategies[511]. MDSCs have been identified in healthy patients[512] but have been demonstrated to be ubiquitously raised in patients with solid malignancies[513] and various inflammatory[514] and pre-malignant conditions[216,515].

In contrast to solid tumours, little is known about MDSC populations or their function in Acute Myeloid Leukaemia (AML). AML is an interesting setting to study immature myeloid cells, given that AML blasts themselves result from the early maturation arrest of the myeloid lineage. Furthermore, it has been suggested that AML blasts exert their suppressive effects on T cells via a similar Arginase-1 dependent mechanism to MDSCs[275]. These observations lead us to investigate the presence and importance of MDSCs in AML and the critical pathways underlying their accumulation and function.

In the present study, AML blasts directed the differentiation of healthy donor PB mononuclear cells towards a cell with the MDSC phenotype (CD11b+, $HLADR^{low/-}$, CD33+). MDSCs from patients with AML and from the *in vitro* co-culture of healthy PBMCs with AML blasts, exhibited immune suppressive activity on co-cultured T cells, as demonstrated by reduced T cell proliferative capacity, reduced expression of markers of activation, reduced IFN-γ production and increased inhibitory IL-10 production.

While these findings are certainly interesting, the model of co-culturing healthy donor PB mononuclear cells with allogeneic AML cells from cells lines or patients with AML has some significant limitations. While cell contact independent and cytokine driven interactions may not require a cell autologous system, one could imagine that cell contact dependent interactions, such as we report, may have a requirement for some level of recognition of self or non self for the full scale of their interaction to be observed *in vitro*. However, an autologous *in vitro* model in AML is near impossible to replicate, as healthy PBMCs in AML are sparse given the overwhelming infiltration of the AML clone in the BM. It is possible that one could isolate AML blasts from a patient and then await a chemotherapy induced remission, but these samples are difficult to come by and patients often have profound cytopoenias for a significant time after induction of remission. Moreover, the majority of patients who enter a true cytogenetic remission then undergo allogeneic transplantation before their counts recover, rendering an autologous system, now an allogeneic one. As such, the allogeneic *in vitro* model that we used for studying MDSCs has been previously published in other disease settings, and our findings were in keeping with other groups, who have reported expansion of MDSCs in similar co-cultures[256]. In an effort to replicate a more physiologically relevant autologous system, we studied the effects of engraftment of syngeneic AML in an immune competent mouse model, demonstrating a significant expansion of MDSCs. In this model, both myeloid compartment and tumour cells are autologous, and their interaction takes place in the BM and spleen, where AML cells home. This most closely mimics the tumour and myeloid compartment interactions that take place in patients with cancer, and is widely published[207].

However murine studies have their own set of limitations, the most fundamental being how well a transplanted cell line can mimic the disease. A single large bolus of tumour cells into the mouse, by retro-orbital injection in our model, must be associated with some degree of local tissue damage and a local inflammatory response which could theoretically mask or indeed exaggerate the expansion of MDSCs seen.

The ability of an AML cell line to home to the bone marrow and engraft is not always predictable or in proportion to the dose of cells given. The described heterogeneity of some cell lines may further confound this. While we have given injected 100,000 AML cells and observe significant populations in the BM after 3 weeks, we do not know if this is a result of one successfully engrafted cell, with stem cell like properties, that then proliferated, or of many cells. If one or some of the cells has some kind of engraftment advantage, this factor could contribute to the degree of MDSC expansion.

Lastly, while C57BL/6 mice were all engrafted at the same day of life and from the same gender and litter, it is a known phenomenon that there is some variability even between litter mates of these heavily inbred strains of mice[516] that may confound the results of some *in vivo* experiments. However, in our experiments, the engraftment of AML resulted in a significant expansion of MDSCs that was seen in all successfully engrafted mice, suggesting that this potential limitation did not affect these experiments.

We have identified MUC1 as a uniquely important oncoprotein in AML and AML stem cells that exerts immunomodulatory effects[128]. We have demonstrated that MUC1 is selectively expressed on AML stem cells as compared to normal haematopoietic stem cells[128]. MUC1 has been shown to exert immunosuppressive effects but the mechanism by which this occurs remains unclear. We sought to elucidate the role of the membrane MUC1 oncoprotein in MDSC expansion. In the present study we have shown that MUC1 silencing in AML, blunts the expansion of MDSCs *in vitro*. We have generated a cell penetrating peptide, GO-203, that disrupts dimerization of the intracellular portion of MUC1-C, preventing its translocation to the nucleus which is critical for downstream signalling. Treatment with the MUC1-C inhibitor GO-203 results in a dose dependent killing of MUC1 expressing cells, including AML cells[129]. Our finding that MUC1 silencing partly abrogates expansion of MDSCs *in vitro*, suggests that treating patients with MUC1 inhibition, might lead to a multi-mechanistic therapeutic benefit, killing MUC1 expressing tumour cells and simultaneously disrupting mechanisms that lead to the accumulation of tumour protecting and immune suppressing MDSCs. To this end, we

have commenced a phase I/II trial of GO-203 in the setting of relapsed-refractory AML[517].

Extracellular vesicles (EVs) are lipid membrane bound vesicles released by cells and mediating inter-cellular communication. It has previously been demonstrated that AML cells release membrane bound extracellular vesicles[412,414,416,436], which transport microRNAs[415], mRNAs[416], cytokines[414] and tumour-derived proteins[412] to surrounding cells. In AML, EVs were shown to export to bystander cells and modify their function in a mRNA and miRNA dependent fashion[416]. In addition, it has been shown in a murine model of breast cancer that tumour EVs skew the BM micro-environment in favour of MDSC accumulation[272] a mechanism dependent on EV inflammatory cytokine cargo. In this study we have demonstrated that AML EVs alter the tumour microenvironment away from antigen presentation capable dendritic cells and towards immature immune suppressive MDSCs. AML EV passage was mostly abrogated by using a 0.4µM Transwell dish, which is in keeping with our previous observation that MDSC expansion was abrogated in Transwell.

The field of EV research is fraught with controversies that may influence how we interpret these experiments. Firstly, are what we are terming EVs actually EVs? The method of isolation of membrane bound vesicles can influence the contents of the resultant fraction heavily. For example, in some of our experiments EVs were precipitated out of solution by SystemBio's ExoQuick[444]. This precipitation method can be somewhat non-specific due to the co-precipitation of other proteins and cell debris. It is therefore possible that the expansion of MDSCs seen upon co-culture of healthy donor PBMCs and AML EVs, could be due to a non-EV contained protein. To control for this possibility, a more elaborate but "cleaner" technique was used in confirmatory experiments, whereby the Qiagen ExoEasy kit, a membrane-based affinity method, was used, which lead to similar results.

Some groups purport that extracellular vesicle contents are not biologically active and are merely a random sample of the cytosol that does not cause biological changes

within up-taking cells. In defence of their relevance, in an intriguing work by Jiminez et al, drug resistance AML cells secreted vesicles carrying anti-apoptotic proteins which were taken up by drug sensitive clones, suggesting TEVs represent a mechanism of the propagation of therapeutic resistance[440]. This would suggest that EVs are indeed a biological relevant entity. As such, the pro-MDSC effects of EVs we have seen in our *in vitro* experiments may well be able to be extrapolated to patients with malignancies.

We subsequently investigated how MUC1 signalling, necessary for the expansion of MDSCs, might alter AML extracellular vesicles composition. We evaluated AML EVs for the presence of the pro-proliferative oncoprotein c-Myc demonstrating that AML cells secrete c-Myc containing EVs in a MUC1 dependent mechanism. Furthermore, MUC1 and c-Myc containing EVs led to an up-regulation of the c-Myc downstream targets cyclin D2 and cyclin E1 in co-cultured MDSCs, indicating that c-Myc containing EVs may drive MDSC proliferation. Critically, EVs from MUC1 silenced AML cells failed to elicit this increase in c-Myc and cyclin D2 and E1 expression in EV exposed MDSCs.

We then sought to determine how MUC1 signalling promotes c-Myc signalling in AML. Micro RNAs are small non-encoding RNA molecules involved in post-translational regulation of gene expression. miR34a, a known p53 inhibitor, has been implicated in regulating the expansion of MDSCs[286] and it is known that tumour cells suppress miR34a expression as part of their self-protective armoury[518]. Furthermore, miR34a is a predicted negative regulator of c-Myc, due to a complementary sequence for miR34a in the c-Myc promoter region.

In the present study, we have demonstrated that MUC1 silencing results in increased expression of miRNA34a. Furthermore, over-expression of miR34a in AML cells led to a dramatic down-regulation of c-Myc, and conversely silencing of miR34a led to a significant up-regulation of c-Myc expression, confirming that miR34a regulates c-Myc expression in AML.

One potential caveat to these experiments is the controversy over how biologically relevant miRNAs are in post-transcriptional regulation of gene expression. While

artificially forcing the silencing or over-expression of miRNAs *in vitro*, can lead to significant translational changes in downstream protein expression, the activity of this pathway in nature is controversial, with some groups purporting that miRNAs are relatively minimally active in regulating gene expression[519]. So while we did observe that over-expression and silencing of miR34a did indeed lead to down-stream changes in c-Myc expression, the inherent role of miR34a in suppressing the translation of c-Myc in healthy myeloid cells has yet to be fully elucidated. In defence of the relevance of this pathway, one group has demonstrated that miR34a is present and moreover critical, in the suppression of proliferation and the promotion of differentiation of myeloid cells in granulopoesis[520]. Although the authors do not investigate this importance of c-Myc in this pathway, they do comment that c-Myc is known to block the function of C/EBPα, which was critical for differentiation in this pathway.

To confirm miR34a as a critical negative regulator of MDSC expansion, miR34a altered cells were interrogated for their ability to elicit an expansion of MDSCs in co-cultured PBMCs. Overexpression of miR34a in AML cells partially abrogated their ability to induce MDSCs from co-cultured donor PBMCs. In concert, silencing of miR34a in MUC1 silenced AML cells, recapitulated their ability to induce MDSCs in this model. Taken together, this study illustrates a hitherto un-described role of the MUC1 oncoprotein in suppressing mechanisms that would otherwise regulate MDSC proliferation and expansion.

In the present study we have shown that patients with active AML have increased numbers of circulating immune suppressive MDSCs. The main difficulty in conducting these experiments was the lack of cell makers that can differentiate an MDSC from a leukaemic blast. For the purposes of these studies, patients were selected for whom clinical flow cytometric analysis had determined a phenotype for the majority of blasts that could differentiate them from CD33+ HLADR- CD11b+ monocytic (CD15-) or granulocytic (CD15+) MDSCs. As an example, an AML sample was determined to be >99% strongly HLADR+, which is sufficiently different from CD33+ HLADR- CD11b+ MDSCs, to allow differentiation of MDSCs and blasts. The limitations of this

methodology are that many samples were discarded due to overlap of AML and MDSC populations, or that there were no differentiating markers. Moreover, the heterogeneity of some samples means that some "MDSCs", may in fact be sub-clones of the AML, which have acquired different cell surface markers. In an attempt to overcome this, we were very stringent about the samples used in analysis, and heterogeneous AML samples were discarded. Having said this, our interpretation of the experiments quantifying MDSCs in AML patients should be considered as preliminary and in need of further validation in larger cohorts than were available here.

While the question of the clonal relationship of MDSCs to AML was beyond the scope of this study, we have begun to explore this in a small cohort of patients. MDSCs isolated by flow cytometric sorting were interrogated for the presence of cytogenetic or molecular abnormalities that had previously been identified in the leukaemic clone. These studies demonstrated diversity with respect to the origin of the MDSC population suggesting they do not exclusively arise from the leukaemic clone. In a patient with AML cells exhibiting 3 cytogenetic abnormalities, (del7, del20, and trisomy 8), MDSCs expressed only del 7 and del20 suggesting a common clonal origin with leukaemic precursor prior to the attainment of the trisomy 8 mutation. In a second patient with del7 AML, only 50% of the MDSCs exhibited the cytogenetic abnormality consistent with mixed derivation from malignant and non-malignant myeloid precursors. In a third patient with AML cells characterized by a NPM1 mutation, the MDSC population was found to have the wild type form of NPM1 consistent with their lack of common clonal derivation with the AML population. These studies suggest that MDSC expansion in the AML microenvironment is not a reflection of myeloid differentiation of the leukaemic clone but rather appears to be an effect on myeloid cells in the bone marrow niche irrespective of their derivation.

The main difficulty in conducting these experiments was the lack of cell makers that can differentiate an MDSC from a leukaemic blast as described above. As AML can demonstrate clonal heterogeneity, it was certainly possible that the "MDSCs" were in

fact a sub-clone of the leukaemic cells, which had further differentiated into a more mature CD15+ and granulocytic phenotype. However the results from the first patient, who's MDSCs harboured in fact one fewer cytogenetic abnormality than the leukaemic blasts, argue against this, as cells may accrue more cytogenetic abnormalities as they undergo clonal evolution or differentiation, but may not lose them. In the third patient with AML cells characterized by a NPM1 mutation, the MDSC population was found to have the wild type form of NPM1 consistent with their lack of common clonal derivation with the AML population. As NPM1 is considered a founder mutation, it seems highly unlikely that the CD15+ MDSCs could be a leukaemic sub-clone that had somehow regained NPM1 wildtype status.

The second patient, who's MDSCs appeared to be a mixed population of clonally related and un-related cells to the leukaemic blasts, raises several possibilities. One interpretation of this experiment is that MDSCs in this patient have a mixed derivation from malignant and non-malignant myeloid precursors. Another, not mutually exclusive, interpretation could be that the "MDSC cells" are in fact two or multiple populations of cells, with different derivations, and our limited markers are pooling multiple potentially very different populations, which may be variably suppressive and as such, not true MDSCs. Ideally, in this patient one would attempt to isolate and functionally compare clonally restricted and related cells but the limitation of cytogenetic analysis is the requirement for fixed and therefore dead cells.

The next question is how in keeping these findings are to other cancer models, where groups have published on the role of MDSCs.

While it has been reported that MDSCs are ubiquitously expanded in patients with cancer[30], including Renal Carcinoma[220]; Melanoma[237]; Prostate[238]; Hepatocellular Carcinoma[239]; Head and neck cancer[240]; Rectal Cancer[92]; Colon and Breast Carcinoma[241]; Glioma[242]; Pancreatic[243] and Non-small cell lung cancer[244], this is the first report detailing the characteristics of MDSCs in AML. Indeed, MDSCs in AML appear to be

comprised of both monocytic and granulocytic subsets, in keeping with the most commonly reported phenotypes of these cells in other human cancer settings.

However, the results of examining the clonality of MDSCs demonstrated that in one of the two patients who had AML with antecedent MDS, the MDSCs appeared to be clonally related to the underlying MDS clone, and not the dominant AML clone. This is in contrast to previous work published in patients with MDS[216], whereby the MDSCs were all clonally distinct from the MDS clone. This disparity could be due to a number of reasons. Firstly, it is possible that the given our small sample size, that our clonality results could be incorrect and due to a technical issue in separating MDSCs, and that subsequent experiments may yield conflicting results. Secondly, it may be that the published MDS study with a sample size of just 5 patients, did not capture the full range of this heterogeneous disease, and it may be that some patients have clonally related, and some clonally distinct subsets of MDSCs. It would be of great interest to perform these examinations of clonality in a large sample of patients, to correlate the clonality of MDSCs with the risk of progression to AML, and see if clonally related or distinct MDSCs exert differing degrees of immune suppression.

One of the most interesting findings from our studies was the observation that *in vitro*, MDSC expansion was abrogated in Transwell experiments, suggesting that the mechanism for MDSC expansion was not due to soluble factors. This is in stark contrast to the body of work in multiple other solid and haematological malignancies, where inflammatory cytokines have been implicated as the main mechanism of tumour induced MDSC expansion. In the present study, we have demonstrated *in vitro* that in AML, the release of EVs, whose passage is abrogated in Transwell, mediates MDSC expansion. There have been, however, several recent reports of extra-cellular vesicles mediating MDSC expansion, in both Breast Cancer[272] and in Melanoma[273] and it may be that as the field of research on EVs becomes more mature, that the breadth of their effects in other cancer settings becomes more apparent.

After demonstrating that MUC1 mediates MDSC expansion by exporting c-Myc containing EVs to nearby cells, we sought to elucidate the mechanism by which MUC1 signalling regulates c-Myc expression. We have demonstrated that MUC1 regulates c-Myc expression in AML cells, and EVs derived from those cells, by repressing miR34a expression. This finding was all the more interesting given that miR34a expression has been previously reported to be involved in the regulation of MDSC expansion in cancer. However in this study the authors postulated that the suppression of MDSC expansion in miR34a over-expressing cells was via the inhibitory effect of miR34a on TGF-B, which were shown to mediate MDSC expansion in their murine model of colon cancer. These two explanations are clearly not mutually exclusive, and as miRNAs have pleomorphic functions in modulating the expression of many genes, it is entirely possible that miR34a may regulates both c-Myc expression and TGF-B secretion in AML, but only c-Myc is active in promoting the proliferation of MDSCs in this disease, where the use of Transwell abrogated MDSC expansion.

We then sought to explain the effect of MUC1 signalling on miR34a. We demonstrated that MUC1 silencing increases miR34a levels, but not levels of its precursor pre-miR34a. This suggested that MUC1 signalling may be interfering with the processing of precursor miRNAs to mature miRNAs. miRNAs are first transcribed as large RNA precursors called pri-miRNAs which are processed in the nucleus by the RNAse III enzymes Drosha and PASHA into pre-miRNAs of roughly 70-nucleotides in length. The pre-miRNAs are exported into the cytoplasm and undergo processing by the RNAse III enzyme Dicer generating the final mature miRNA, which consists of double-stranded RNA of roughly 22 nucleotides in length[34,35]. Following this processing, DICER bound mature miRNA forms a complex known as the RNA-induced silencing complex (RISC), made up of DICER, miRNA, a transport protein called transactivating response RNA-binding protein (TRBP) and Argonaut2 (Ago2). TRBP recruits Ago-2 to the RISC, whereby it serves to cleave the target mRNA strand complementary to their bound miRNA[36]. We interrogated MUC1 silenced and MUC1 inhibitor treated AML cells for DICER and Argonaut2 expression, demonstrating that MUC1 silencing increases the expression of DICER. Furthermore, we

determined that MUC1 down-regulates DICER expression by transcriptional repression of the transcription factor c-JUN. Given the observed effect of MUC1 on miRNA processing machinery we sought to determine if multiple miRNA species were affected by MUC1 regulation of DICER expression. Indeed miRNA array of MUC1 silenced AML cell lines MOLM-14 and THP-1 demonstrated an increase in the majority of mRNAs in AML cells, reaching Bonferroni-corrected significance in a subset.

These data raise many interesting questions. Firstly, it is known that DICER activity mediates maturation of miRNA in all but one known miRNAs (miR-451). As such, why does the increase in DICER expression seen upon MUC1 silencing only increase a subset of miRNAs arrayed, and not all except miR-451, whose maturation is dependent on Argonaut2 alone[521]? The answer may lie in the technical limitations of the microarray used. The calculation of fold change in miRNA expression and the testing of the change for statistical significance is strongly affected by the relative abundance of individual miRNAs, which is widely variable. As such, a small increase in expression of a particular miRNA, which does not reach statistical significance because of low baseline abundance, may be much more biologically active than another miRNA with a significant fold change in expression. As such, data from miRNA-arrays should be interpreted with caution, and observations must be validated with quantitative PCR and functional tests of miRNA activity, such as with luciferase based reporter assays. While it was beyond the scope of this study to perform qPCR and reporter assays on 800+ miRNAs, the broad trend of miRNAs increasing upon MUC1 silencing is most provocative.

Interestingly, while the levels of miR-451 were unchanged upon MUC1 silencing of MOLM-14 cells, they were significantly increased in MUC1 silenced THP-1 cells, compared to control. If miR-451 were truly DICER independent, one would expect the expression to remain unchanged upon MUC1 silencing. While it is possible reports of its DICER-independent processing may be flawed, another explanation is that miR-451 is also epigenetically regulated. There is increasing evidence that MUC1 is involved in hypermethylation of key genes in human cancer[503,522] and it is known that certain

miRNAs have CpG islands within their promoter sequences and are, as such, subject to epigenetic regulation[523]. While there does not appear to be a CpG island within 2kb of the miR-451 sequence, there is a report of miR-451 increasing upon treatment with a hypomethylating agent[524], and furthermore, in an Alzheimer's disease model, miR-451 did indeed appear to be hyper-methylated[525].

In order to ascertain the role, if any, of hyper-methylation in MUC1s regulation of cJUN and DICER expression, we treated AML cells with a sub-lethal dose of the HMA Decitabine, and observed an increase in the expression of cJUN and DICER with a resultant reduction in c-Myc expression. We may infer that the reduction of c-Myc observed is a result of the hypomethylation of entities upstream of c-Myc, as hypomethylating increases protein expression, rather than decreases it.

While these experiments suggest a role for hypomethylation in explaining the effect of MUC1 silencing on downstream signalling, the evidence is somewhat circumstantial and further work is needed to delineate the relative contributions of transcription factor driven vs. epigenetic changes observed after MUC1 silencing or inhibition.

Lastly, we attempted to exploit our improved understanding of these critical signalling pathways in order to target MDSCs. Using a small peptide inhibitor of MUC1 signalling (GO-203) and a novel hypomethylating agent (SGI-110), we demonstrated a decrease in MDSC accumulation *in vitro* and *in vivo*. In the experiments using MUC1 inhibition, the reduction in MDSCs seen, could not be fully attributed to a direct effect on MDSCs, as small (and non-significant) reduction in tumour burden was also seen, which may also lead to a reduction in expansion in MDSCs. While this limits our interpretation of this experiment, it offers up an exciting translational opportunity, to target both the tumour cell and surrounding immune suppressive stromal milieu.

We subsequently demonstrated that hypomethylation using a novel hypomethylating agent SGI-110, significantly reduces the AML induced expansion of MDSCs. Indeed, the reduction in MDSC burden was more dramatic than in the studies using MUC1

inhibition. Furthermore, in the *in vivo* model of murine AML, MDSC depletion occurred by day 16, several days before tumour burden began to fall. This suggests a more direct effect of hypomethylation on MDSCs, independent of tumour burden, and might lead us to hypothesize that the trend towards a reduction in tumour burden in SGI-110 treated animals seen at day 19, might be in part mediated by a reduction in immune suppressive MDSCs, perhaps leading to greater immune cell mediated anti-tumour cytotoxicity.

AML is a lethal haematologic malignancy for which curative outcomes remain elusive. A major area of investigation lies in developing novel immunotherapeutic strategies to selectively target the tumour cell. Elucidating the role of MDSCs in AML may further our understanding of the critical molecular pathways that hamper immune responses to immunotherapy. In the present study we have demonstrated that MDSCs are expanded in patients with AML and contribute to tumour related immune suppression. We have shown that the MUC1 oncoprotein is a critical mediator of MDSC expansion, via the promotion of c-Myc expression in secreted EVs. Finally we demonstrate that MUC1 regulation of c-Myc is mediated by the repression of the maturation of the microRNA miR34a, occurring via the down-regulation of DICER expression. MUC1s effect on DICER expression results in a global down-regulation of miRNA-genesis, mediated by transcription and epigenetic effects.

Strategies that combine tumour-specific targeting while simultaneously reversing the immune suppressive milieu, for example by targeting MDSCs, represent an innovative approach to the treatment of AML, potentially changing the paradigm of how AML is treated by targeting both the tumour and the tumour supporting microenvironment.

Future Work

In the last chapter we demonstrated that hypomethylation using a novel hypomethylating agent SGI-110, significantly reduces the AML induced expansion of MDSCs *in vitro* and *in vivo*. Furthermore, in the *in vivo* model of murine AML, MDSC depletion occurred by day 16, several days before tumour burden began to fall. This suggested a direct effect of hypomethylation on MDSCs, independent of tumour burden and might lead us to hypothesize that the trend towards a reduction in tumour burden in SGI-110 treated animals seen at day 19, might be in part mediated by a reduction in immune suppressive MDSCs, perhaps leading to greater immune cell mediated anti-tumour cytotoxicity. To investigate this hypothesis, the same murine experiment will be performed in NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ NSG mice, who lack a functioning T cell compartment but retain the majority of their myeloid compartment containing MDSCs. If SGI-110 treated mice do not demonstrate the same reduction in tumour burden post MDSC depletion, we might conclude that the anti-tumour effects of SGI-110 are immune mediated. To confirm this, the T cell compartment will be "added back" to the mice to see if tumour depletion then occurs. However if SGI-110 causes the same pattern of MDSC depletion followed by tumour depletion in NSG mice, then we might conclude that the effect of SGI-110 is purely cytotoxic and MDSCs are more sensitive to this and are depleted earlier than AML cells. Further studies will also be performed to look at DICER and miRNA expression in AML cells harvested from SGI-110 treated tumour bearing mice, to determine if DICER up-regulation is the critical mediator of the anti-tumour and anti-MDSC effects of hypomethylation with SGI-110.

Lastly, synergy studies, combining targeting of tumour cells with immune based therapies and depleting MDSCs will be undertaken to determine if targeting MDSCs can improve responses to immune based therapies. Our group has developed a novel dendritic cell (DC) AML cell fusion vaccine in which patient derived leukaemia cells are fused with autologous dendritic cells such that a broad array of tumour associated antigens are presented in the context of DC mediated co-stimulation. In an ongoing

phase II clinical trial, AML patients who achieve remission following standard cytotoxic therapy underwent serial vaccination with DC/AML fusions. Vaccination resulted in the dramatic induction of leukaemia specific immunity, as measured by a mean fold increase of CD4 and CD8 cells expressing IFN-γ in response to *ex vivo* exposure to autologous leukaemia cell lysates and the associated expansion of T cells in the peripheral blood and bone marrow targeting the AML antigens MUC1, WT1 and Pr1. Remarkably, despite an average age of 61, 75% of patients (12/16) undergoing vaccination remain in sustained remission with a median follow up of 33 months. We hypothesize that suppression of MDSC expansion in AML will help to reverse the immunosuppressive environment of the bone marrow microenvironment creating an enhanced platform for AML vaccination. Targeting MDSCs using SGI-110, could increase immunological responses to treatment with DC/tumour fusion vaccine.

Appendix A: Companies

Company	**Address**
Abcam	1 Kendall Square, Suite B2304 Cambridge, MA 02139-1517 USA
Addgene	Addgene 75 Sidney St #550A, Cambridge, MA 02139
Affymetrix	Affymetrix 3420 Central Expy, Santa Clara, CA 95051
American Type Culture Collection	American Type Culture Collection P.O. Box 1549 Manassas, VA 20108 USA
BD Biosciences	BD Biosciences 2350 Qume Drive San Jose, CA 95131 877.232.8995
Beckman Coulter	Beckman Coulter

	36 Cherry Hill Dr, Danvers, MA 01923
Berlex Laboratories	Berlex Laboratories 37 Terry Ave Edison, New Jersey 08820- 3929 United States
Biolegend	Biolegend 9727 Pacific Heights Blvd. San Diego, CA 92121
Cell Signalling	Cell Signalling 3 Trask Lane Danvers, MA, 01923 United States
Cellgenix	Cellgenix Am Flughafen 16, 79108 Freiburg im Breisgau, Germany
Cellgro (Mediatech)	Cellgro (Mediatech) 9345 Discovery Blvd, Manassas, VA 20109

Corning	Corning One Riverfront Plaza Corning, NY, 14831, USA USA
DAKO	Dako North America, Inc. 6392 Via Real Carpinteria, CA 93013
eBioscience	eBioscience 10255 Science Center Dr, San Diego, CA 92121
GE Healthcare	GE Healthcare Little Chalfont, Buckinghamshire, United Kingdom
Genome Engineering Production Group	Harvard Medical School, Boston MA USA
Invitrogen,	1600 Faraday Avenue

	PO Box 6482, Carlsbad CA, 92008
Invivogen	Invivogen 3950 Sorrento Valley Blvd, Suite 100 San Diego, California 92121 USA
Jackson Lab	Jackson Lab 600 Main Street Bar Harbor, ME USA 04609
JEOL USA Inc.	JEOL USA Inc. 11 Dearborn Rd, Peabody, MA 01960
Kraft Family Blood Donor Center	Dana Farber Cancer Institute, 35 Binney St, Boston, MA 02115 USA
LabScientific, Inc	LabScientific, Inc 114 W Mt Pleasant Ave # A, Livingston, NJ 07039
NanoString	NanoString

	530 Fairview Ave N, Seattle, WA 98109
OncoImmunin	OncoImmunin 207 Perry Pkwy #6, Gaithersburg, MD 20877
Promega	Promega 2800 Woods Hollow Rd, Fitchburg, WI 53711
Qiagen, Germany	QIAGEN Strasse 1 40724 Hilden, Germany
R&D	R&D 614 McKinley Place NE Minneapolis, MN 55413
Sigma	Sigma PO Box 14508 St. Louis, MO 63178 USA
System Biosciences	System Biosciences , 2438 Embarcadero Way, Palo Alto, CA 94303

Tecan, Mennedorf,	Tecan Group Ltd. Seestrasse 103. 8708 Männedorf, Switzerland.
The Jackson Laboratory	The Jackson Laboratory 600 Main St, Bar Harbor, ME 04609
ThermoScientific	ThermoScientific 790 Memorial Dr, Cambridge, MA 02139
Vysis (Abbott Molecular)	Abbott Molecular 1300 E Touhy Ave Des Plaines, IL 60018

Appendix B: Antibodies

Antibody	Clone	Isotype	Company	Catalogue No.
CD11b-APCCy7	M1/70	Rat IgG2b, κ	Biolegend	101226
CD33-PE	P67.6	Mouse IgG_1, κ	BDBioScience	340679
HLADR-FITC	L243	IgG_2, κ	BDBioScience	347363
CD14-PB	HCD14	Mouse IgG1, κ	Biolegend	325615
CD15-APC	HI98	Mouse IgM, κ	Biolegend	301907
mGr1-APC	RB6-8C5	Rat IgG2b, κ	Biolegend	108411
CD3-FITC	UCHT1	Mouse IgG1, kappa	eBioscience	11-0038-41
CD69-PE	L78	Mouse IgG_1, κ	BDBioScience	341652
CD25-FITC	2A3	Mouse IgG_1, κ	BDBioScience	340694
CD4-PB	GK1.5	Rat IgG2b, κ	Biolegend	100438
CD8-FITC	SK1	Mouse IgG_1, κ	eBioscience	9011-0087
IFN-γ-PE	B27	Mouse / IgG1, kappa	Invitrogen	MHCIFG04-3

IL-10-PE	JES3-9D7	Rat / IgG1, kappa	eBioscience	RHCIL1004
MUC1-C	MH1 (CT2)	Armenian hamster / IgG	Thermo Scientific	MA5-11202
c-Myc	Y69	Rabbit IgG	Abcam	32072
CD63	EPR5702	Rabbit IgG	Abcam	ab134045
B-actin	D6A8	Rabbit IgG	Cell Signalling	8457S
Cyclin E1	HE12	Mouse IgG1	Cell Signalling	4129P
Cyclin D2	D52F9	Rabbit IgG	Cell Signalling	3741S
GAPDH	14C10	Rabbit IgG	Cell Signalling	2118S
DICER	D38E7	Rabbit IgG	Cell Signalling	5362
Argo2	C34C6	Rabbit IgG	Cell Signalling	2897P
cJUN	60A8	Rabbit IgG	Cell Signalling	9165P
phospho-cjUN	Ser63	Rabbit IgG	Cell Signalling	2361P
NPM1	376	IgG1	DAKO	IR65261
anti-mouse secondary	-	-	DAKO	Z042001-2

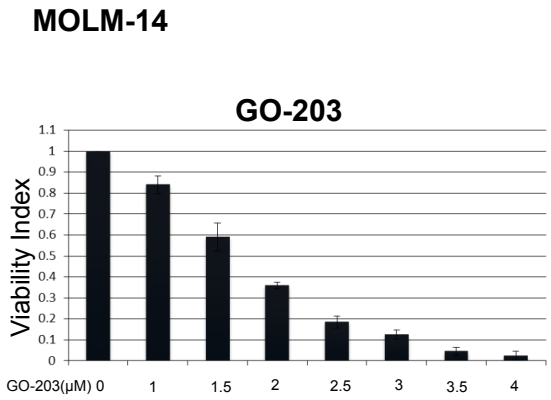
antibody				

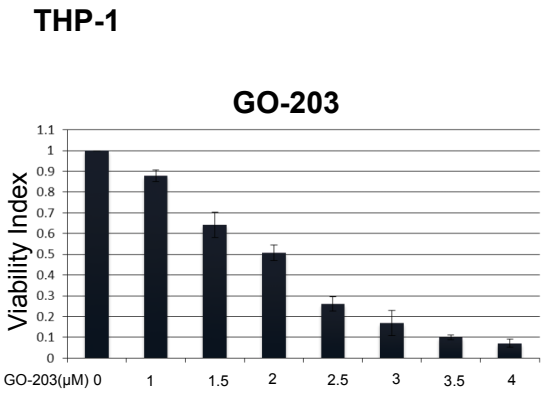
Appendix C: PCR Primers

Primer	Company	Catalogue No.
18s	Qiagen	QF00451850
b-actin	Qiagen	QT01680476
c-myc	Qiagen	QT00035406
DICER	Qiagen	QT00015176
GAPDH	Qiagen	QT00079247
JUN	Qiagen	QT00242956
miR34a	Qiagen	MS00003318
precursor-miR34a	Qiagen	MP00002044

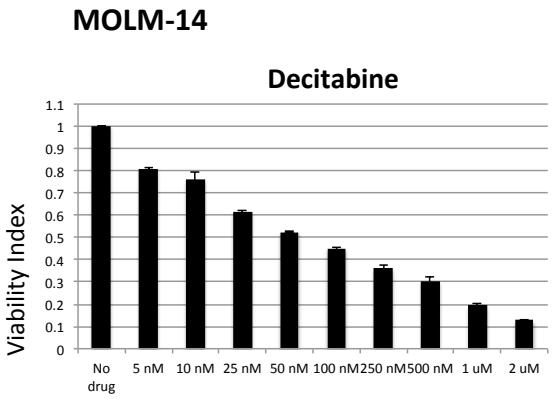
Appendix D: Drug Dose Curves

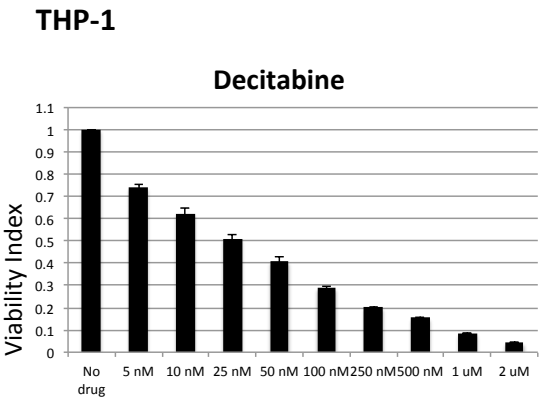
1. MUC1 inhibitor (GO-203)





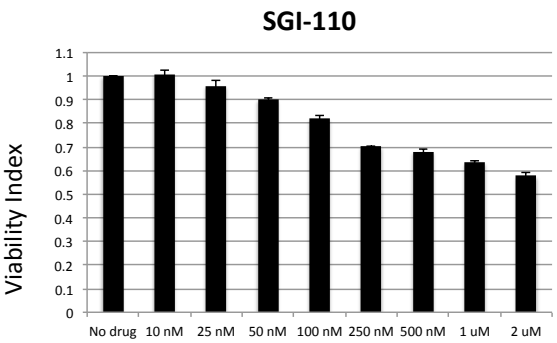
2. Decitabine



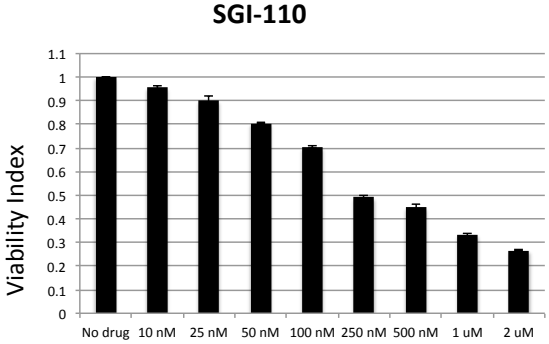


3. SGI-110

MOLM-14


SGI-110
Viability Index
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
No drug
10 nM
25 nM
50 nM
100 nM
250 nM
500 nM
1 uM
2 uM

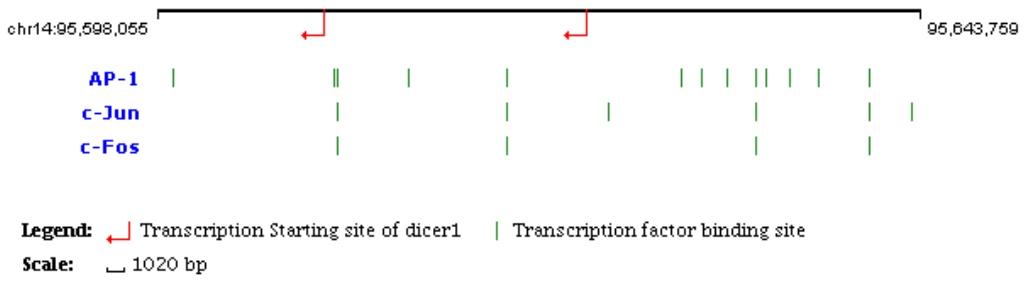
THP-1


SGI-110
Viability Index
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
No drug
10 nM
25 nM
50 nM
100 nM
250 nM
500 nM
1 uM
2 uM

Appendix E: Transcription Factor binding sites

1. Prediction of Transcription Factors with binding site homology in the DICER promoter

Software used: Champion ChiP Transcription Factor Search Portal [SABiosciences]



2. Prediction of miRNAs with binding site homology in the 3'UTR for c-Myc

Software used: microRNA.org

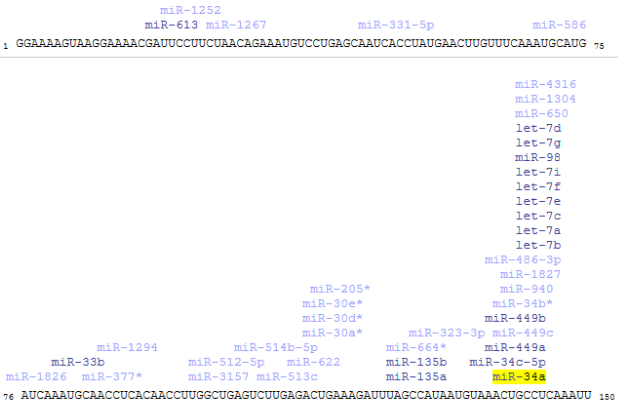
hsa-miR-34a/MYC Alignment

```
    3' ugUUGGUCGAUU-C-UGUGACGGu 5' hsa-miR-34a
       |:|||  ||| | | ||||||
123:5' uuAGCCA--UAAUGUAAACUGCCu 3' MYC
```

mirSVR score: -0.1630
PhastCons score: 0.6594

Mouseover a miRNA mature name to see the miRNA/MYC alignment.

MYC v-myc myelocytomatosis viral oncogene homolog (avian)



Appendix F: Absolute Frequencies of Cells in Figures 5,9,15,16

Figure 5b (%MDSCs)		No tumour	MOLM-14	THP-1	AML Patient
	Sample a	2.07	7.8	10	4.02
	Sample b	2.9	4.2	9	3.08
	Sample c	1.1	6	11	5
Figure 5c (%MDSCs)		PBMCs	PBMCs + MOLM-14	PBMCs + MOLM-14 (Transwell)	
	Sample a	6	31	10	
	Sample b	8	21	12	
	Sample c	6	30	8	
Figure 9a (Luminescence, Relative Light Units)		Tc	Tc + THP-1 MDSC (2:1)	Tc + MOLM-14 MDSC (2:1)	
	Sample a	58678	39826	18373	
	Sample b	59010	29873	18273	
	Sample c	66741	32983	16353	
Figure 9b (%CD8+IFNY+)		Tc	Tc + CD3/28	Tc + CD3/28 + MOLM-14 MDSC (2:1)	
	Sample a	1	3	1.7	
	Sample b	1.2	4.9	4.2	

	Sample c	3	5.8	0.1	
Figure 9c (%CD4+, CD25+, CD69+)		Tc	Tc + CD3/28	Tc + CD3/28 + MOLM-14 MDSC (2:1)	
	Sample a	8	12	2	
	Sample b	0.2	0.8	0.4	
	Sample c	0.1	0.9	0.6	

Figure 9d (%CD4+IL-10+)		Tc	Tc + CD3/28	Tc + CD3/28 + MOLM-14 MDSC (2:1)	
	Sample a	0.21	0.4	3	
	Sample b	0.4	0.01	6	
	Sample c	0.1	0.1	1	
Figure 15a (%MDSCs)		PBMCs	PBMCs +MOLM-14 EVs	PBMCs +MOLM-14 EVs (Transwell)	
	Sample a	0.2	3.1	1.1	
	Sample b	1.7	6	1.9	
	Sample c	1.1	5.2	3.1	
Figure 15b (% CD11c+, HLADR+ APCs)		PBMCs	PBMCs +MOLM-14 EVs	PBMCs +MOLM-14 EVs (Transwell)	
	Sample a	0.2	0.01	0.12	
	Sample b	1.7	0.04	1	
	Sample c	1.1	0.2	0.7	
Figure 16c (%MDSCs)		MOLM-14 control shRNA	MOLM-14 MUC-1 shRNA	THP-1 control shRNA	THP-1 MUC-1 shRNA
	Sample a	31.05	6.5	4.8	1.82

	Sample b	15	10	9.05	7.23
	Sample c	3	1.2	12.8	8.18
Figure 16d (%MDSCs)		PBMCs	PBMCs +MOLM-14	PBMCs +MOLM-14 MUC1 CRISPR/Cas9	
	Sample a	7	25	16	
	Sample b	2	12	4	
	Sample c	2	15	1	

Appendix G: Data Sets (mRNA and miRNA arrays)

1. mRNA Array

adj.P.Val	SYMBOL	FoldChange
8.20E-09	RN7SK	3.03070728
0.000258035	RNU1-5	2.151581152
0.00039884	RNU1G2	1.868155269
0.00056599	RNU4ATAC	4.089101983
0.003231965	LOC648927	0.664340218
0.003231965	RNU1-3	1.933928896
0.003231965	JUN	2.215131546

2. microRNA Array

Cell Line: MOLM-14

MiRNA	Fold change after MUC1 silencing
hsa-miR-150-5p	13.45
hsa-miR-548aa+hsa-miR-548t-3p	9.26
hsa-miR-122-5p	7.47
hsa-miR-125a-3p	7.16
hsa-miR-520d-3p	6.78
hsa-miR-877-5p	6.75
hsa-miR-16-5p	6.35
hsa-miR-219a-2-3p	5.91
hsa-miR-549a	5.13
hsa-miR-4443	5.12
hsa-miR-1290	4.89
hsa-miR-944	4.86
hsa-miR-146b-5p	4.65
hsa-miR-644a	4.59
hsa-miR-2116-5p	4.46
hsa-miR-548n	4.39
hsa-miR-4536-5p	4.36
hsa-miR-522-3p	4.34
hsa-miR-30e-5p	4.32
hsa-miR-548j-5p	4.31
hsa-miR-320c	4.21

hsa-miR-494-5p	4.14
hsa-miR-26a-5p	3.86
hsa-miR-302a-5p	3.86
hsa-miR-2113	3.83
hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518e-5p+hsa-miR-522-5p+hsa-miR-519a-5p	3.79
hsa-miR-1266-5p	3.73
hsa-miR-4516	3.73
hsa-miR-3140-3p	3.72
hsa-miR-374c-5p	3.7
hsa-miR-128-2-5p	3.66
hsa-miR-152-3p	3.64
hsa-miR-4421	3.61
hsa-miR-483-3p	3.59
hsa-miR-561-5p	3.59
hsa-miR-6720-3p	3.59
hsa-miR-380-3p	3.57
hsa-miR-103a-3p	3.51
hsa-miR-520f-3p	3.51
hsa-miR-181a-5p	3.49
hsa-miR-504-5p	3.49
hsa-miR-548j-3p	3.47
hsa-miR-219b-3p	3.4

hsa-miR-496	3.4
hsa-miR-591	3.38
hsa-miR-4455	3.37
hsa-miR-342-3p	3.35
hsa-miR-1269a	3.34
hsa-miR-1295a	3.33
hsa-miR-216b-5p	3.33
hsa-miR-651-3p	3.33
hsa-miR-383-5p	3.32
hsa-miR-2117	3.3
hsa-miR-4531	3.3
hsa-miR-146b-3p	3.26
hsa-miR-375	3.26
hsa-miR-374a-3p	3.25
hsa-miR-3136-5p	3.2
hsa-miR-320b	3.2
hsa-miR-875-3p	3.19
hsa-miR-182-5p	3.17
hsa-miR-556-5p	3.17
hsa-miR-451a	3.16
hsa-miR-548a-3p	3.16
hsa-miR-136-5p	3.15
hsa-miR-362-3p	3.15

hsa-miR-548g-3p	3.14
hsa-miR-4647	3.13
hsa-miR-1250-5p	3.12
hsa-miR-152-5p	3.11
hsa-miR-21-5p	3.11
hsa-miR-24-3p	3.09
hsa-miR-328-3p	3.07
hsa-miR-194-5p	3.06
hsa-miR-1236-3p	3.04
hsa-miR-4435	3.02
hsa-miR-526b-5p	3.02
hsa-miR-767-3p	3.02
hsa-miR-1249-3p	3
hsa-miR-532-3p	3
hsa-miR-590-5p	3
hsa-miR-1244	2.98
hsa-miR-1262	2.98
hsa-miR-133b	2.98
hsa-miR-424-5p	2.98
hsa-miR-619-3p	2.98
hsa-miR-1287-3p	2.97
hsa-miR-196a-5p	2.97
hsa-miR-4454+hsa-miR-7975	2.96

hsa-miR-542-3p	2.96
hsa-miR-577	2.96
hsa-miR-92a-1-5p	2.96
hsa-miR-578	2.95
hsa-miR-124-3p	2.94
hsa-miR-489-3p	2.94
hsa-miR-515-3p	2.93
hsa-miR-19b-3p	2.92
hsa-miR-30b-5p	2.92
hsa-miR-6511a-5p	2.92
hsa-miR-1323	2.91
hsa-miR-1469	2.9
hsa-miR-509-3p	2.9
hsa-miR-4461	2.88
hsa-miR-555	2.87
hsa-miR-208a-3p	2.86
hsa-miR-211-5p	2.86
hsa-miR-29b-3p	2.86
hsa-miR-1298-5p	2.85
hsa-miR-650	2.85
hsa-miR-1273c	2.84
hsa-miR-544a	2.84
hsa-miR-652-5p	2.84

hsa-miR-1247-5p	2.83
hsa-miR-3130-3p	2.83
hsa-miR-892a	2.82
hsa-miR-939-5p	2.82
hsa-miR-3614-5p	2.81
hsa-miR-567	2.81
hsa-miR-188-5p	2.8
hsa-miR-203a-5p	2.8
hsa-miR-376a-3p	2.8
hsa-miR-599	2.8
hsa-miR-874-3p	2.8
hsa-miR-1271-5p	2.79
hsa-miR-3613-3p	2.79
hsa-miR-539-5p	2.79
hsa-miR-3182	2.78
hsa-miR-574-5p	2.77
hsa-miR-582-3p	2.77
hsa-miR-183-5p	2.76
hsa-miR-654-5p	2.75
hsa-miR-509-3-5p	2.74
hsa-miR-598-3p	2.74
hsa-miR-605-5p	2.74
hsa-miR-1202	2.73

hsa-miR-548a-5p	2.73
hsa-miR-769-3p	2.73
hsa-miR-3614-3p	2.71
hsa-miR-421	2.71
hsa-miR-552-3p	2.71
hsa-miR-575	2.71
hsa-miR-1206	2.7
hsa-miR-200a-3p	2.7
hsa-miR-3934-5p	2.7
hsa-miR-1827	2.69
hsa-miR-1302	2.68
hsa-miR-600	2.68
hsa-miR-615-3p	2.68
hsa-miR-873-5p	2.68
hsa-miR-10a-5p	2.67
hsa-miR-1291	2.66
hsa-miR-548i	2.66
hsa-miR-548l	2.66
hsa-miR-3150b-3p	2.65
hsa-miR-329-3p	2.65
hsa-miR-499a-3p	2.65
hsa-miR-518e-3p	2.65
hsa-miR-654-3p	2.65

hsa-miR-301b-3p	2.64
hsa-miR-4451	2.64
hsa-miR-1297	2.63
hsa-miR-1537-3p	2.63
hsa-miR-425-5p	2.63
hsa-miR-132-3p	2.62
hsa-miR-323b-3p	2.62
hsa-miR-484	2.62
hsa-miR-517a-3p	2.62
hsa-miR-593-3p	2.62
hsa-miR-764	2.62
hsa-miR-2053	2.61
hsa-miR-448	2.61
hsa-miR-127-5p	2.6
hsa-miR-135a-5p	2.6
hsa-miR-219a-5p	2.6
hsa-miR-187-3p	2.59
hsa-miR-1908-3p	2.59
hsa-miR-891b	2.59
hsa-miR-149-5p	2.58
hsa-miR-27b-3p	2.58
hsa-miR-486-3p	2.58
hsa-miR-1271-3p	2.57

hsa-miR-186-5p	2.57
hsa-miR-1973	2.57
hsa-miR-432-5p	2.57
hsa-miR-302e	2.56
hsa-miR-3161	2.56
hsa-miR-572	2.56
hsa-miR-100-5p	2.55
hsa-miR-106b-5p	2.55
hsa-miR-1322	2.55
hsa-miR-378h	2.55
hsa-miR-758-3p+hsa-miR-411-3p	2.55
hsa-miR-200b-3p	2.54
hsa-miR-574-3p	2.54
hsa-miR-576-5p	2.54
hsa-miR-630	2.54
hsa-miR-23c	2.53
hsa-miR-499b-5p	2.53
hsa-miR-92b-3p	2.53
hsa-miR-1204	2.52
hsa-miR-1268b	2.52
hsa-miR-1277-3p	2.52
hsa-miR-1306-3p	2.52
hsa-miR-29c-3p	2.52

hsa-miR-556-3p	2.51
hsa-miR-642a-5p	2.51
hsa-miR-671-3p	2.51
hsa-miR-210-5p	2.49
hsa-miR-219a-1-3p	2.49
hsa-miR-3144-5p	2.49
hsa-miR-524-3p	2.49
hsa-miR-548ai+hsa-miR-570-5p	2.49
hsa-miR-548z+hsa-miR-548h-3p	2.49
hsa-miR-7-5p	2.49
hsa-miR-107	2.48
hsa-miR-140-3p	2.48
hsa-miR-323a-5p	2.48
hsa-miR-585-3p	2.48
hsa-miR-1255b-5p	2.47
hsa-miR-151b	2.47
hsa-miR-30a-5p	2.47
hsa-miR-2278	2.46
hsa-miR-30c-5p	2.46
hsa-miR-1268a	2.45
hsa-miR-128-1-5p	2.45
hsa-miR-518d-3p	2.45
hsa-miR-520a-5p	2.45

hsa-miR-548ah-5p	2.45
hsa-miR-1248	2.44
hsa-miR-337-5p	2.43
hsa-miR-548h-5p	2.43
hsa-miR-876-3p	2.43
hsa-miR-1255a	2.42
hsa-miR-1257	2.42
hsa-miR-153-3p	2.42
hsa-miR-31-5p	2.42
hsa-miR-4431	2.42
hsa-miR-1185-2-3p	2.41
hsa-miR-346	2.41
hsa-miR-1224-3p	2.4
hsa-miR-3065-5p	2.4
hsa-miR-373-3p	2.4
hsa-miR-508-3p	2.4
hsa-miR-517b-3p	2.4
hsa-miR-617	2.4
hsa-miR-640	2.39
hsa-miR-1178-3p	2.38
hsa-miR-454-3p	2.38
hsa-miR-499a-5p	2.38
hsa-miR-513b-5p	2.38

hsa-miR-139-5p	2.37
hsa-miR-378d	2.37
hsa-miR-1301-3p	2.36
hsa-miR-130b-3p	2.36
hsa-miR-1915-3p	2.36
hsa-miR-302f	2.36
hsa-miR-4524a-5p	2.36
hsa-miR-4792	2.36
hsa-miR-487b-5p	2.36
hsa-miR-614	2.36
hsa-miR-626	2.36
hsa-miR-942-5p	2.36
hsa-miR-148b-3p	2.35
hsa-miR-26b-5p	2.35
hsa-miR-411-5p	2.35
hsa-miR-562	2.35
hsa-miR-134-3p	2.33
hsa-miR-512-3p	2.33
hsa-miR-516a-5p	2.33
hsa-miR-205-5p	2.32
hsa-miR-32-5p	2.32
hsa-miR-4521	2.32
hsa-miR-98-3p	2.32

hsa-miR-1972	2.31
hsa-miR-199a-3p+hsa-miR-199b-3p	2.31
hsa-miR-326	2.31
hsa-miR-378g	2.31
hsa-miR-501-3p	2.31
hsa-miR-660-5p	2.31
hsa-miR-193b-3p	2.3
hsa-miR-518b	2.3
hsa-miR-606	2.29
hsa-miR-185-5p	2.28
hsa-miR-324-5p	2.28
hsa-miR-3605-5p	2.28
hsa-miR-1200	2.27
hsa-miR-151a-3p	2.27
hsa-miR-3190-3p	2.27
hsa-miR-342-5p	2.27
hsa-miR-4425	2.27
hsa-miR-520d-5p+hsa-miR-527+hsa-miR-518a-5p	2.27
hsa-miR-548q	2.27
hsa-miR-210-3p	2.26
hsa-miR-218-5p	2.26
hsa-miR-1908-5p	2.25
hsa-miR-195-5p	2.25

hsa-miR-324-3p	2.25
hsa-miR-409-5p	2.25
hsa-miR-147b	2.24
hsa-miR-498	2.24
hsa-miR-514b-3p	2.24
hsa-miR-302b-3p	2.23
hsa-miR-1307-5p	2.22
hsa-miR-198	2.22
hsa-miR-302c-3p	2.22
hsa-miR-450b-5p	2.22
hsa-miR-25-5p	2.21
hsa-miR-323a-3p	2.21
hsa-miR-363-5p	2.21
hsa-miR-365a-3p+hsa-miR-365b-3p	2.21
hsa-miR-429	2.21
hsa-miR-490-3p	2.21
hsa-miR-525-3p	2.21
hsa-miR-193a-5p+hsa-miR-193b-5p	2.2
hsa-miR-196a-3p	2.2
hsa-miR-296-5p	2.2
hsa-miR-511-5p	2.2
hsa-miR-761	2.2
hsa-miR-941	2.2

hsa-miR-23a-3p	2.19
hsa-miR-545-3p	2.19
hsa-miR-548b-3p	2.19
hsa-miR-3192-5p	2.18
hsa-miR-487b-3p	2.18
hsa-miR-509-5p	2.18
hsa-miR-548e-3p	2.18
hsa-miR-96-5p	2.18
hsa-miR-433-3p	2.17
hsa-miR-519e-3p	2.17
hsa-miR-664a-3p	2.17
hsa-miR-1-5p	2.16
hsa-miR-3127-5p	2.16
hsa-miR-3928-3p	2.16
hsa-miR-874-5p	2.16
hsa-miR-500a-5p+hsa-miR-501-5p	2.15
hsa-miR-548ad-3p	2.15
hsa-miR-548d-3p	2.15
hsa-miR-643	2.15
hsa-miR-655-3p	2.15
hsa-miR-142-3p	2.14
hsa-miR-144-3p	2.14
hsa-miR-208b-3p	2.14

hsa-miR-221-3p	2.14
hsa-miR-2682-5p	2.14
hsa-miR-3195	2.14
hsa-miR-345-3p	2.14
hsa-miR-376a-2-5p	2.14
hsa-miR-182-3p	2.13
hsa-miR-214-3p	2.13
hsa-miR-335-5p	2.13
hsa-miR-339-3p	2.13
hsa-miR-4284	2.13
hsa-miR-455-5p	2.13
hsa-miR-936	2.13
hsa-miR-1281	2.12
hsa-miR-181c-5p	2.12
hsa-miR-211-3p	2.12
hsa-miR-323b-5p	2.12
hsa-miR-4707-5p	2.12
hsa-miR-548ak	2.12
hsa-miR-652-3p	2.12
ACTB	2.12
hsa-miR-34c-5p	2.11
hsa-miR-374a-5p	2.11
hsa-miR-384	2.11

hsa-miR-566	2.11
hsa-miR-885-3p	2.11
hsa-miR-3690	2.1
hsa-miR-5010-5p	2.1
hsa-miR-506-3p	2.1
hsa-miR-6721-5p	2.1
hsa-miR-888-5p	2.1
hsa-miR-22-3p	2.09
hsa-miR-298	2.09
hsa-miR-29a-3p	2.09
hsa-miR-330-5p	2.09
hsa-miR-584-3p	2.09
hsa-miR-639	2.09
hsa-miR-10b-5p	2.08
hsa-miR-1185-5p	2.08
hsa-miR-302a-3p	2.08
hsa-miR-30a-3p	2.08
hsa-miR-3202	2.08
hsa-miR-362-5p	2.08
hsa-miR-550a-5p	2.08
hsa-miR-1193	2.07
hsa-miR-1264	2.07
hsa-miR-1289	2.07

hsa-miR-216a-5p	2.07
hsa-miR-382-3p	2.07
hsa-miR-493-3p	2.07
hsa-miR-1260a	2.06
hsa-miR-193a-3p	2.06
hsa-miR-221-5p	2.06
hsa-miR-301a-5p	2.06
hsa-miR-379-5p	2.06
hsa-miR-637	2.06
hsa-miR-137	2.05
hsa-miR-485-5p	2.05
hsa-miR-892b	2.05
hsa-miR-25-3p	2.04
hsa-miR-3185	2.04
hsa-miR-520g-3p	2.04
hsa-miR-573	2.04
hsa-miR-6511a-3p	2.04
hsa-miR-1245b-3p	2.03
hsa-miR-1304-5p	2.03
hsa-miR-133a-5p	2.03
hsa-miR-190a-5p	2.03
hsa-miR-450b-3p	2.03
hsa-miR-542-5p	2.03

hsa-miR-620	2.03
hsa-miR-627-3p	2.03
hsa-miR-1279	2.02
hsa-miR-381-5p	2.02
hsa-miR-4286	2.02
hsa-miR-576-3p	2.02
hsa-miR-887-3p	2.02
hsa-miR-1203	2.01
hsa-miR-1276	2.01
hsa-miR-891a-5p	2.01
hsa-miR-940	2.01
hsa-miR-98-5p	2.01
hsa-miR-1249-5p	2
hsa-miR-1253	2
hsa-miR-128-3p	2
hsa-miR-1283	2
hsa-miR-143-3p	2
hsa-miR-18b-5p	2
hsa-miR-302d-3p	2
hsa-miR-3144-3p	2
hsa-miR-378e	2
hsa-miR-422a	2
hsa-miR-4755-5p	2

hsa-miR-494-3p	2
hsa-miR-502-5p	2
hsa-miR-548ar-5p	2
hsa-miR-570-3p	2
hsa-miR-579-3p	2
hsa-miR-603	2
hsa-miR-610	2
hsa-miR-627-5p	2
hsa-miR-744-5p	2
hsa-miR-1299	1.99
hsa-miR-138-5p	1.99
hsa-miR-200c-3p	1.99
hsa-miR-217	1.99
hsa-miR-3613-5p	1.99
hsa-miR-518f-3p	1.99
hsa-miR-532-5p	1.99
hsa-miR-587	1.99
hsa-miR-1285-3p	1.98
hsa-miR-129-5p	1.98
hsa-miR-320e	1.98
hsa-miR-553	1.98
hsa-miR-885-5p	1.98
hsa-let-7f-5p	1.97

hsa-miR-1183	1.97
hsa-miR-1306-5p	1.97
hsa-miR-18a-5p	1.97
hsa-miR-204-5p	1.97
hsa-miR-28-3p	1.97
hsa-miR-331-5p	1.97
hsa-miR-4787-5p	1.97
hsa-miR-548k	1.97
hsa-miR-126-3p	1.96
hsa-miR-497-5p	1.96
hsa-miR-508-5p	1.96
hsa-miR-597-5p	1.96
hsa-miR-934	1.96
hsa-miR-133a-3p	1.95
hsa-miR-1910-5p	1.95
hsa-miR-376b-3p	1.95
hsa-miR-376c-5p	1.95
hsa-miR-499b-3p	1.95
hsa-miR-519d-3p	1.95
hsa-miR-520h	1.95
hsa-miR-548e-5p	1.95
hsa-miR-628-3p	1.95
hsa-miR-770-5p	1.95

hsa-miR-1270	1.94
hsa-miR-184	1.94
hsa-miR-371b-5p	1.94
hsa-miR-503-3p	1.94
hsa-miR-760	1.94
hsa-miR-1252-5p	1.93
hsa-miR-301b-5p	1.93
hsa-miR-34b-3p	1.93
hsa-miR-571	1.93
hsa-miR-589-5p	1.93
hsa-miR-641	1.92
hsa-miR-6503-5p	1.92
hsa-miR-1305	1.91
hsa-miR-142-5p	1.91
hsa-miR-4707-3p	1.91
hsa-miR-514a-3p	1.91
hsa-miR-561-3p	1.91
hsa-miR-582-5p	1.91
hsa-miR-648	1.91
hsa-miR-651-5p	1.91
hsa-miR-802	1.91
hsa-miR-181b-5p+hsa-miR-181d-5p	1.9
hsa-miR-3151-5p	1.9

hsa-miR-328-5p	1.9
hsa-miR-452-5p	1.9
hsa-miR-4532	1.9
hsa-miR-890	1.9
hsa-miR-1976	1.89
hsa-miR-222-3p	1.89
hsa-miR-523-3p	1.89
hsa-miR-551b-3p	1.89
hsa-miR-592	1.89
hsa-miR-660-3p	1.89
hsa-miR-92a-3p	1.89
hsa-miR-146a-5p	1.88
hsa-miR-188-3p	1.88
hsa-miR-3168	1.88
hsa-miR-331-3p	1.88
hsa-miR-338-5p	1.88
hsa-miR-5001-3p	1.88
hsa-miR-1197	1.87
hsa-miR-3147	1.87
hsa-miR-320a	1.87
hsa-miR-490-5p	1.87
hsa-miR-507	1.87
hsa-miR-876-5p	1.87

hsa-miR-154-5p	1.86
hsa-miR-181a-3p	1.86
hsa-miR-1205	1.85
hsa-miR-1286	1.85
hsa-miR-15a-5p	1.85
hsa-miR-3180-3p	1.85
hsa-miR-455-3p	1.85
hsa-miR-513c-5p	1.85
hsa-miR-889-3p	1.85
hsa-miR-1185-1-3p	1.84
hsa-miR-1303	1.84
hsa-miR-147a	1.84
hsa-miR-181a-2-3p	1.84
hsa-miR-28-5p	1.84
hsa-miR-377-3p	1.84
hsa-miR-4741	1.84
hsa-miR-580-3p	1.84
hsa-miR-224-5p	1.83
hsa-miR-3158-3p	1.83
hsa-miR-541-3p	1.83
hsa-miR-612	1.83
hsa-miR-192-5p	1.82
hsa-miR-370-5p	1.82

hsa-miR-381-3p	1.82
hsa-miR-873-3p	1.82
hsa-miR-3074-3p	1.81
hsa-miR-3615	1.81
hsa-miR-367-3p	1.81
hsa-miR-378i	1.81
hsa-miR-431-5p	1.81
hsa-miR-510-3p	1.81
hsa-miR-1909-3p	1.8
hsa-miR-30e-3p	1.8
hsa-miR-4458	1.8
hsa-miR-450a-5p	1.8
hsa-miR-513a-5p	1.8
hsa-miR-595	1.8
hsa-miR-1260b	1.79
hsa-miR-206	1.79
hsa-miR-4485-3p	1.79
hsa-miR-564	1.79
hsa-miR-624-3p	1.79
hsa-miR-1245a	1.78
hsa-miR-223-3p	1.78
hsa-miR-378c	1.78
hsa-miR-371a-5p	1.77

hsa-miR-548m	1.77
hsa-let-7c-5p	1.76
hsa-miR-20a-5p+hsa-miR-20b-5p	1.76
hsa-miR-33b-5p	1.76
hsa-miR-369-3p	1.76
hsa-miR-374b-5p	1.76
hsa-miR-568	1.76
hsa-miR-767-5p	1.76
hsa-miR-9-5p	1.76
hsa-miR-409-3p	1.75
hsa-miR-638	1.75
hsa-miR-937-3p	1.75
hsa-miR-1287-5p	1.74
hsa-miR-140-5p	1.74
hsa-miR-3164	1.74
hsa-miR-584-5p	1.74
hsa-miR-933	1.74
hsa-miR-433-5p	1.73
hsa-miR-495-3p	1.73
hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	1.73
hsa-miR-769-5p	1.73
hsa-miR-95-3p	1.73
hsa-miR-3180-5p	1.72

hsa-miR-412-3p	1.72
hsa-miR-6503-3p	1.72
hsa-miR-1-3p	1.71
hsa-miR-300	1.71
hsa-miR-369-5p	1.71
hsa-miR-299-5p	1.7
hsa-miR-512-5p	1.7
hsa-miR-516b-5p	1.7
hsa-miR-519b-3p	1.7
hsa-miR-548y	1.7
hsa-miR-579-5p	1.7
hsa-miR-99b-5p	1.7
hsa-miR-127-3p	1.69
hsa-miR-215-5p	1.69
hsa-miR-320d	1.69
hsa-miR-505-3p	1.69
hsa-miR-520b	1.69
hsa-miR-642a-3p	1.69
hsa-miR-758-5p	1.69
hsa-miR-1293	1.68
hsa-miR-1307-3p	1.68
hsa-miR-382-5p	1.68
hsa-miR-3918	1.68

hsa-miR-521	1.68
hsa-miR-1261	1.67
hsa-miR-1272	1.67
hsa-miR-199a-5p	1.67
hsa-miR-199b-5p	1.67
hsa-miR-299-3p	1.67
hsa-miR-449c-5p	1.67
hsa-miR-519c-3p	1.67
hsa-miR-3196	1.66
hsa-miR-4448	1.66
hsa-miR-4787-3p	1.66
hsa-miR-491-5p	1.66
hsa-miR-5010-3p	1.66
hsa-miR-543	1.66
hsa-miR-604	1.66
hsa-miR-765	1.66
hsa-miR-27a-3p	1.65
hsa-miR-510-5p	1.65
hsa-miR-516a-3p+hsa-miR-516b-3p	1.65
hsa-miR-631	1.65
hsa-miR-1245b-5p	1.64
hsa-miR-518c-3p	1.64
hsa-miR-671-5p	1.64

hsa-miR-23b-3p	1.63
hsa-miR-296-3p	1.63
hsa-miR-297	1.63
hsa-miR-197-5p	1.62
hsa-miR-212-3p	1.62
hsa-miR-551a	1.62
hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p	1.61
hsa-miR-935	1.61
hsa-miR-1910-3p	1.6
hsa-miR-202-3p	1.6
hsa-miR-548v	1.6
hsa-miR-596	1.6
hsa-miR-661	1.6
hsa-miR-1296-3p	1.59
hsa-miR-190a-3p	1.59
hsa-miR-378f	1.59
hsa-miR-5196-5p	1.59
hsa-miR-608	1.59
hsa-miR-6724-5p	1.59
hsa-miR-1234-3p	1.58
hsa-miR-125a-5p	1.58
hsa-miR-515-5p	1.58
hsa-miR-548d-5p	1.58

hsa-miR-628-5p	1.58
hsa-miR-766-3p	1.58
hsa-miR-125b-5p	1.57
hsa-miR-139-3p	1.57
hsa-miR-34c-3p	1.57
hsa-miR-601	1.57
hsa-miR-656-3p	1.57
hsa-miR-181b-2-3p	1.56
hsa-miR-363-3p	1.56
hsa-miR-491-3p	1.56
hsa-miR-502-3p	1.56
hsa-miR-625-5p	1.55
hsa-miR-1258	1.54
hsa-miR-1285-5p	1.54
hsa-miR-330-3p	1.54
hsa-miR-607	1.54
hsa-miR-181d-3p	1.53
hsa-miR-203a-3p	1.53
hsa-miR-301a-3p	1.53
hsa-miR-339-5p	1.53
hsa-miR-101-3p	1.52
hsa-miR-105-5p	1.52
hsa-miR-34a-5p	1.52

hsa-miR-361-3p	1.52
hsa-miR-488-3p	1.52
hsa-miR-504-3p	1.52
hsa-miR-663a	1.52
hsa-miR-1233-3p	1.51
hsa-miR-135b-5p	1.51
hsa-miR-370-3p	1.51
hsa-miR-145-5p	1.5
hsa-miR-155-5p	1.5
hsa-miR-5001-5p	1.5
hsa-miR-554	1.5
hsa-miR-99a-5p	1.5
hsa-miR-1275	1.49
hsa-miR-141-3p	1.49
hsa-miR-337-3p	1.49
hsa-miR-514b-5p	1.49
hsa-miR-1226-3p	1.48
hsa-miR-196b-5p	1.48
hsa-miR-665	1.48
hsa-miR-922	1.48
hsa-miR-1254	1.47
hsa-miR-1304-3p	1.47
hsa-miR-487a-3p	1.47

hsa-miR-548al	1.47
hsa-miR-376c-3p	1.46
hsa-miR-664b-5p	1.46
hsa-miR-325	1.45
hsa-miR-5196-3p+hsa-miR-6732-3p	1.44
hsa-miR-151a-5p	1.43
hsa-miR-30d-5p	1.43
hsa-miR-3140-5p	1.43
hsa-miR-1180-3p	1.42
hsa-miR-410-3p	1.42
hsa-miR-450a-2-3p	1.42
hsa-miR-520e	1.42
hsa-miR-539-3p	1.42
hsa-miR-649	1.42
hsa-miR-675-5p	1.42
hsa-miR-361-5p	1.41
hsa-miR-450a-1-3p	1.41
hsa-miR-485-3p	1.4
hsa-miR-664b-3p	1.4
hsa-miR-423-5p	1.39
hsa-miR-4536-3p	1.39
hsa-miR-483-5p	1.39
hsa-miR-514a-5p	1.39

hsa-miR-520c-3p	1.39
hsa-miR-590-3p	1.39
hsa-miR-129-2-3p	1.38
hsa-miR-345-5p	1.38
hsa-miR-513a-3p	1.38
hsa-miR-525-5p	1.38
hsa-miR-197-3p	1.37
hsa-miR-340-5p	1.37
hsa-miR-1278	1.36
hsa-miR-1296-5p	1.36
hsa-miR-517c-3p+hsa-miR-519a-3p	1.36
hsa-miR-329-5p	1.35
hsa-miR-372-3p	1.35
hsa-miR-423-3p	1.35
hsa-miR-924	1.35
hsa-let-7e-5p	1.34
hsa-miR-1288-3p	1.34
hsa-miR-942-3p	1.34
hsa-miR-548ar-3p	1.33
hsa-miR-1269b	1.32
hsa-miR-148a-3p	1.31
hsa-miR-19a-3p	1.3
hsa-miR-3180	1.3

hsa-miR-887-5p	1.3
hsa-miR-3131	1.29
hsa-miR-449b-5p	1.29
hsa-miR-563	1.29
hsa-miR-616-3p	1.29
hsa-miR-629-5p	1.29
hsa-miR-3605-3p	1.27
hsa-miR-513c-3p	1.26
hsa-miR-613	1.26
hsa-miR-495-5p	1.25
hsa-miR-190b	1.22
hsa-miR-208b-5p	1.22
hsa-miR-1224-5p	1.21
hsa-miR-506-5p	1.2
hsa-miR-520a-3p	1.19
hsa-miR-2110	1.16
hsa-let-7b-5p	1.14
hsa-miR-615-5p	1.14
hsa-miR-3916	1.13
hsa-miR-708-5p	1.13
hsa-let-7g-5p	1.12
hsa-miR-492	1.12
hsa-miR-548c-5p+hsa-miR-548o-5p+hsa-miR-548am-5p	1.11

hsa-miR-106a-5p+hsa-miR-17-5p	1.08
hsa-miR-130a-3p	1.05
hsa-miR-378b	1.04
hsa-miR-33a-5p	1.03
hsa-miR-3065-3p	1.02
hsa-miR-449a	1.01
hsa-miR-766-5p	-1.01
hsa-miR-503-5p	-1.04
hsa-miR-1228-3p	-1.06
hsa-miR-191-5p	-1.07
hsa-miR-365b-5p	-1.07
hsa-miR-134-5p+hsa-miR-6728-5p	-1.14
hsa-miR-15b-5p	-1.16
hsa-let-7i-5p	-1.18
hsa-miR-3179	-1.27
hsa-let-7d-5p	-1.3
hsa-miR-93-5p	-1.39
hsa-miR-4488	-1.54
B2M	-1.62
hsa-let-7a-5p	-5.82
hsa-miR-1246	-5.97

Cell Line: THP-1

MiRNA	Fold change after MUC1 silencing
hsa-miR-196a-5p	11.69
hsa-miR-512-3p	4.04
hsa-miR-3140-3p	3.89
hsa-miR-664a-3p	3.79
hsa-miR-1180-3p	3.51
hsa-miR-378c	3.25
hsa-miR-370-5p	3.19
hsa-miR-3140-5p	3.17
GAPDH	3.14
hsa-miR-525-5p	3.01
hsa-miR-503-3p	2.98
hsa-miR-1910-3p	2.79
hsa-miR-6503-3p	2.76
hsa-miR-744-5p	2.73
hsa-miR-649	2.66
hsa-miR-181b-2-3p	2.59
hsa-miR-19a-3p	2.59
hsa-miR-3180	2.56
hsa-miR-509-5p	2.55
hsa-miR-532-5p	2.54
hsa-miR-18b-5p	2.53
hsa-miR-2117	2.52

hsa-miR-182-3p	2.5
hsa-miR-3164	2.49
hsa-miR-550a-5p	2.47
hsa-miR-369-5p	2.45
hsa-miR-1302	2.42
hsa-miR-4421	2.4
hsa-miR-539-3p	2.39
hsa-miR-103a-3p	2.38
hsa-miR-506-5p	2.37
hsa-miR-196a-3p	2.35
hsa-miR-3074-3p	2.35
hsa-miR-520g-3p	2.35
hsa-miR-876-3p	2.35
hsa-miR-664b-3p	2.31
hsa-miR-665	2.3
hsa-miR-372-3p	2.28
hsa-miR-380-3p	2.28
hsa-miR-4741	2.28
hsa-miR-708-5p	2.28
hsa-miR-3180-3p	2.23
hsa-miR-29a-3p	2.22
hsa-miR-33b-5p	2.22
hsa-miR-942-3p	2.22

hsa-miR-548v	2.21
hsa-miR-3144-5p	2.2
hsa-miR-376a-2-5p	2.2
hsa-miR-487b-3p	2.2
hsa-miR-520b	2.2
hsa-miR-891a-5p	2.2
hsa-miR-3613-5p	2.18
hsa-miR-671-5p	2.18
hsa-miR-548l	2.17
hsa-miR-202-3p	2.15
hsa-miR-511-5p	2.15
hsa-miR-330-5p	2.14
hsa-miR-6503-5p	2.14
hsa-miR-3180-5p	2.13
hsa-miR-320c	2.13
hsa-miR-208b-5p	2.12
hsa-miR-144-3p	2.11
hsa-miR-769-3p	2.1
hsa-miR-382-5p	2.09
hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	2.09
hsa-miR-615-5p	2.09
hsa-miR-296-3p	2.08
hsa-miR-1301-3p	2.06

hsa-miR-210-5p	2.05
hsa-miR-340-5p	2.05
hsa-miR-641	2.05
hsa-miR-937-3p	2.05
hsa-miR-203a-5p	2.04
hsa-miR-302e	2.03
hsa-miR-373-3p	2.03
hsa-miR-3615	2.02
hsa-miR-369-3p	2.02
hsa-miR-1249-5p	2.01
hsa-miR-133a-5p	2.01
hsa-miR-34c-3p	2.01
hsa-miR-519d-3p	2.01
hsa-miR-544a	2.01
hsa-miR-664b-5p	2.01
hsa-miR-887-3p	2.01
hsa-miR-1291	2
hsa-miR-514a-3p	2
hsa-miR-597-5p	2
hsa-miR-190a-5p	1.99
hsa-miR-302a-5p	1.99
hsa-miR-378f	1.99
hsa-miR-409-5p	1.99

hsa-miR-140-5p	1.98
hsa-miR-181d-3p	1.98
hsa-miR-568	1.98
hsa-miR-652-3p	1.98
hsa-miR-561-3p	1.97
hsa-miR-215-5p	1.96
hsa-miR-299-3p	1.96
hsa-miR-7-5p	1.96
hsa-miR-204-5p	1.94
hsa-miR-367-3p	1.94
hsa-miR-449b-5p	1.94
hsa-miR-624-3p	1.94
hsa-miR-331-5p	1.93
hsa-miR-589-5p	1.93
hsa-miR-892b	1.93
hsa-miR-1304-3p	1.92
hsa-miR-1537-3p	1.92
hsa-miR-513a-3p	1.92
hsa-miR-1281	1.91
hsa-miR-508-3p	1.91
hsa-miR-571	1.91
hsa-miR-580-3p	1.91
hsa-miR-4536-3p	1.9

hsa-miR-1289	1.89
hsa-miR-296-5p	1.89
hsa-miR-516a-3p+hsa-miR-516b-3p	1.89
hsa-miR-197-5p	1.88
hsa-miR-3127-5p	1.88
hsa-miR-616-3p	1.88
hsa-miR-628-5p	1.88
hsa-miR-1254	1.87
hsa-miR-136-5p	1.87
hsa-miR-885-3p	1.87
hsa-miR-146b-3p	1.86
hsa-miR-3131	1.86
hsa-miR-323b-5p	1.86
hsa-miR-496	1.86
hsa-miR-548m	1.86
hsa-miR-592	1.86
hsa-miR-1185-1-3p	1.85
hsa-miR-1288-3p	1.85
hsa-miR-554	1.85
hsa-miR-615-3p	1.85
hsa-miR-660-5p	1.85
hsa-miR-205-5p	1.84
hsa-miR-1268b	1.83

hsa-miR-1270	1.83
hsa-miR-363-5p	1.83
hsa-miR-4431	1.83
hsa-miR-4461	1.83
hsa-miR-567	1.83
hsa-miR-628-3p	1.83
hsa-miR-892a	1.83
hsa-miR-1197	1.82
hsa-miR-1287-3p	1.82
hsa-miR-301b-3p	1.82
hsa-miR-1261	1.81
hsa-miR-1296-3p	1.81
hsa-miR-151a-5p	1.81
hsa-miR-218-5p	1.81
hsa-miR-221-5p	1.81
hsa-miR-512-5p	1.81
hsa-miR-520c-3p	1.81
hsa-miR-548j-5p	1.81
hsa-miR-922	1.81
hsa-miR-1245b-5p	1.8
hsa-miR-3202	1.8
hsa-miR-577	1.8
hsa-miR-638	1.8

hsa-miR-944	1.8
hsa-miR-206	1.79
hsa-miR-382-3p	1.79
hsa-miR-4516	1.79
hsa-miR-518b	1.79
hsa-miR-128-2-5p	1.78
hsa-miR-411-5p	1.78
hsa-miR-181a-2-3p	1.77
hsa-miR-3150b-3p	1.77
hsa-miR-370-3p	1.77
hsa-miR-450a-2-3p	1.77
hsa-miR-516b-5p	1.77
hsa-miR-1183	1.76
hsa-miR-1185-2-3p	1.76
hsa-miR-153-3p	1.76
hsa-miR-378b	1.76
hsa-miR-6511a-5p	1.76
hsa-miR-555	1.75
hsa-miR-626	1.75
hsa-miR-145-5p	1.74
hsa-miR-455-5p	1.74
hsa-miR-542-3p	1.74
hsa-miR-556-5p	1.74

hsa-miR-598-3p	1.74
hsa-miR-643	1.74
hsa-miR-1285-3p	1.73
hsa-miR-329-3p	1.73
hsa-miR-3168	1.72
hsa-miR-548h-5p	1.72
hsa-miR-548k	1.72
hsa-miR-1278	1.71
hsa-miR-182-5p	1.71
hsa-miR-192-5p	1.71
hsa-miR-483-3p	1.71
hsa-miR-489-3p	1.71
hsa-miR-523-3p	1.71
hsa-miR-889-3p	1.71
hsa-miR-939-5p	1.71
hsa-miR-323a-3p	1.7
hsa-miR-338-5p	1.7
hsa-miR-520d-3p	1.7
hsa-miR-548a-3p	1.7
hsa-miR-590-5p	1.7
hsa-miR-1272	1.69
hsa-miR-25-5p	1.69
hsa-miR-337-3p	1.69

hsa-miR-452-5p	1.69
hsa-miR-4787-5p	1.69
hsa-miR-490-3p	1.69
hsa-miR-216b-5p	1.68
hsa-miR-383-5p	1.68
hsa-miR-4451	1.68
hsa-miR-4531	1.68
hsa-miR-150-5p	1.67
hsa-miR-2682-5p	1.67
hsa-miR-508-5p	1.67
hsa-miR-548j-3p	1.67
hsa-miR-342-5p	1.66
hsa-miR-345-5p	1.66
hsa-miR-556-3p	1.66
hsa-miR-1226-3p	1.65
hsa-miR-1262	1.65
hsa-miR-1915-3p	1.65
hsa-miR-330-3p	1.65
hsa-miR-381-3p	1.65
hsa-miR-3934-5p	1.65
hsa-miR-495-5p	1.65
hsa-miR-601	1.65
hsa-miR-10a-5p	1.64

hsa-miR-1264	1.64
hsa-miR-195-5p	1.64
hsa-miR-4286	1.64
hsa-miR-6721-5p	1.64
hsa-miR-148a-3p	1.63
hsa-miR-379-5p	1.63
hsa-miR-500a-5p+hsa-miR-501-5p	1.63
hsa-miR-518c-3p	1.63
hsa-miR-532-3p	1.63
hsa-miR-1299	1.62
hsa-miR-18a-5p	1.62
hsa-miR-299-5p	1.62
hsa-miR-519e-3p	1.62
hsa-miR-563	1.62
hsa-miR-6720-3p	1.62
hsa-miR-1224-5p	1.61
hsa-miR-1297	1.61
hsa-miR-302b-3p	1.61
hsa-miR-384	1.61
hsa-miR-4647	1.61
hsa-miR-517c-3p+hsa-miR-519a-3p	1.61
hsa-miR-593-3p	1.61
hsa-miR-758-3p+hsa-miR-411-3p	1.61

hsa-miR-211-3p	1.6
hsa-miR-412-3p	1.6
hsa-miR-551b-3p	1.6
hsa-miR-219a-1-3p	1.59
hsa-miR-320a	1.59
hsa-miR-519c-3p	1.59
hsa-miR-96-5p	1.59
hsa-miR-1258	1.58
hsa-miR-127-5p	1.58
hsa-miR-1279	1.58
hsa-miR-149-5p	1.58
hsa-miR-31-5p	1.58
hsa-miR-3179	1.58
hsa-miR-320d	1.58
hsa-miR-325	1.58
hsa-miR-506-3p	1.58
hsa-miR-548i	1.58
hsa-miR-637	1.58
hsa-miR-1185-5p	1.57
hsa-miR-1287-5p	1.57
hsa-miR-1305	1.57
hsa-miR-323b-3p	1.57
hsa-miR-449a	1.57

hsa-miR-4532	1.57
hsa-miR-485-5p	1.57
hsa-miR-498	1.57
hsa-miR-509-3-5p	1.57
hsa-miR-1271-3p	1.56
hsa-miR-28-5p	1.56
hsa-miR-490-5p	1.56
hsa-miR-599	1.56
hsa-miR-625-5p	1.56
hsa-miR-6511a-3p	1.56
hsa-miR-132-3p	1.55
hsa-miR-152-3p	1.55
hsa-miR-409-3p	1.55
hsa-miR-4755-5p	1.55
hsa-miR-484	1.55
hsa-miR-518f-3p	1.55
hsa-miR-675-5p	1.55
hsa-miR-1200	1.54
hsa-miR-1236-3p	1.54
hsa-miR-2116-5p	1.54
hsa-miR-301a-5p	1.54
hsa-miR-433-5p	1.54
hsa-miR-510-3p	1.54

hsa-miR-541-3p	1.54
hsa-miR-566	1.54
hsa-miR-219a-2-3p	1.53
hsa-miR-27a-3p	1.53
hsa-miR-302a-3p	1.53
hsa-miR-3605-5p	1.53
hsa-miR-424-5p	1.53
hsa-miR-1306-3p	1.52
hsa-miR-193b-3p	1.52
hsa-miR-196b-5p	1.52
hsa-miR-23c	1.52
hsa-miR-3613-3p	1.52
hsa-miR-378g	1.52
hsa-miR-518e-3p	1.52
hsa-miR-524-3p	1.52
hsa-miR-549a	1.52
hsa-miR-663a	1.52
hsa-miR-942-5p	1.52
hsa-miR-1245a	1.51
hsa-miR-1275	1.51
hsa-miR-193a-3p	1.51
hsa-miR-335-5p	1.51
hsa-miR-365a-3p+hsa-miR-365b-3p	1.51

hsa-miR-200a-3p	1.5
hsa-miR-2113	1.5
hsa-miR-374a-3p	1.5
hsa-miR-449c-5p	1.5
hsa-miR-548q	1.5
hsa-miR-552-3p	1.5
hsa-miR-576-3p	1.5
hsa-miR-758-5p	1.5
hsa-miR-92a-1-5p	1.5
hsa-miR-1245b-3p	1.49
hsa-miR-134-3p	1.49
hsa-miR-3196	1.49
hsa-miR-4485-3p	1.49
hsa-miR-4536-5p	1.49
hsa-miR-584-5p	1.49
hsa-miR-612	1.49
hsa-miR-1252-5p	1.48
hsa-miR-126-3p	1.48
hsa-miR-1266-5p	1.48
hsa-miR-1469	1.48
hsa-miR-29c-3p	1.48
hsa-miR-362-3p	1.48
hsa-miR-374c-5p	1.48

hsa-miR-429	1.48
hsa-miR-4488	1.48
hsa-miR-513a-5p	1.48
hsa-miR-585-3p	1.48
hsa-miR-619-3p	1.48
hsa-miR-766-5p	1.48
hsa-miR-105-5p	1.47
hsa-miR-1244	1.47
hsa-miR-1306-5p	1.47
hsa-miR-208b-3p	1.47
hsa-miR-302f	1.47
hsa-miR-3185	1.47
hsa-miR-3190-3p	1.47
hsa-miR-324-3p	1.47
hsa-miR-374a-5p	1.47
hsa-miR-3928-3p	1.47
hsa-miR-504-5p	1.47
hsa-miR-548a-5p	1.47
hsa-miR-1228-3p	1.46
hsa-miR-1268a	1.46
hsa-miR-140-3p	1.46
hsa-miR-183-5p	1.46
hsa-miR-184	1.46

hsa-miR-1910-5p	1.46
hsa-miR-200b-3p	1.46
hsa-miR-3161	1.46
hsa-miR-339-5p	1.46
hsa-miR-376c-5p	1.46
hsa-miR-425-5p	1.46
hsa-miR-640	1.46
hsa-miR-1193	1.45
hsa-miR-147b	1.45
hsa-miR-200c-3p	1.45
hsa-miR-28-3p	1.45
hsa-miR-302c-3p	1.45
hsa-miR-487a-3p	1.45
hsa-miR-5010-3p	1.45
hsa-miR-503-5p	1.45
hsa-miR-514b-3p	1.45
hsa-miR-548z+hsa-miR-548h-3p	1.45
hsa-miR-551a	1.45
hsa-miR-99a-5p	1.45
hsa-miR-139-3p	1.44
hsa-miR-211-5p	1.44
hsa-miR-497-5p	1.44
hsa-miR-654-3p	1.44

hsa-miR-346	1.43
hsa-miR-548al	1.43
hsa-miR-553	1.43
hsa-miR-605-5p	1.43
hsa-miR-651-5p	1.43
hsa-miR-891b	1.43
hsa-miR-124-3p	1.42
hsa-miR-1249-3p	1.42
hsa-miR-1909-3p	1.42
hsa-miR-217	1.42
hsa-miR-219b-3p	1.42
hsa-miR-337-5p	1.42
hsa-miR-371b-5p	1.42
hsa-miR-513c-5p	1.42
hsa-miR-629-5p	1.42
RPL19	1.42
hsa-miR-1202	1.41
hsa-miR-141-3p	1.41
hsa-miR-363-3p	1.41
hsa-miR-590-3p	1.41
hsa-miR-650	1.41
hsa-miR-765	1.41
hsa-miR-181c-5p	1.4

hsa-miR-2110	1.4
hsa-miR-26b-5p	1.4
hsa-miR-30a-3p	1.4
hsa-miR-3195	1.4
hsa-miR-32-5p	1.4
hsa-miR-365b-5p	1.4
hsa-miR-510-5p	1.4
hsa-miR-513b-5p	1.4
hsa-miR-760	1.4
hsa-miR-934	1.4
hsa-miR-1286	1.39
hsa-miR-1290	1.39
hsa-miR-1293	1.39
hsa-miR-19b-3p	1.39
hsa-miR-4425	1.39
hsa-miR-5001-5p	1.39
hsa-miR-587	1.39
hsa-miR-627-3p	1.39
hsa-miR-648	1.39
hsa-miR-1260b	1.38
hsa-miR-154-5p	1.38
hsa-miR-301b-5p	1.38
hsa-miR-381-5p	1.38

hsa-miR-520e	1.38
hsa-miR-548ai+hsa-miR-570-5p	1.38
hsa-miR-614	1.38
hsa-miR-631	1.38
hsa-miR-339-3p	1.37
hsa-miR-548c-5p+hsa-miR-548o-5p+hsa-miR-548am-5p	1.37
hsa-miR-608	1.37
hsa-miR-873-5p	1.37
hsa-miR-151b	1.36
hsa-miR-1973	1.36
hsa-miR-214-3p	1.36
hsa-miR-331-3p	1.36
hsa-miR-345-3p	1.36
hsa-miR-4455	1.36
hsa-miR-483-5p	1.36
hsa-miR-548ad-3p	1.36
hsa-miR-613	1.36
hsa-miR-887-5p	1.36
hsa-miR-146b-5p	1.35
hsa-miR-186-5p	1.35
hsa-miR-3147	1.35
hsa-miR-329-5p	1.35
hsa-miR-933	1.35

hsa-miR-122-5p	1.34
hsa-miR-1246	1.34
hsa-miR-1253	1.34
hsa-miR-1277-3p	1.34
hsa-miR-128-3p	1.34
hsa-miR-1283	1.34
hsa-miR-143-3p	1.34
hsa-miR-152-5p	1.34
hsa-miR-199a-3p+hsa-miR-199b-3p	1.34
hsa-miR-302d-3p	1.34
hsa-miR-3144-3p	1.34
hsa-miR-378e	1.34
hsa-miR-422a	1.34
hsa-miR-494-3p	1.34
hsa-miR-502-5p	1.34
hsa-miR-505-3p	1.34
hsa-miR-519b-3p	1.34
hsa-miR-548aa+hsa-miR-548t-3p	1.34
hsa-miR-548ar-5p	1.34
hsa-miR-570-3p	1.34
hsa-miR-579-3p	1.34
hsa-miR-603	1.34
hsa-miR-627-5p	1.34

hsa-miR-767-5p	1.34
hsa-miR-185-5p	1.33
hsa-miR-219a-5p	1.33
hsa-miR-30b-5p	1.33
hsa-miR-30e-5p	1.33
hsa-miR-3690	1.33
hsa-miR-518d-3p	1.33
hsa-miR-542-5p	1.33
hsa-miR-548e-5p	1.33
hsa-miR-764	1.33
hsa-miR-873-3p	1.33
hsa-miR-1205	1.32
hsa-miR-29b-3p	1.32
hsa-miR-539-5p	1.32
hsa-miR-561-5p	1.32
hsa-miR-582-5p	1.32
hsa-miR-1178-3p	1.31
hsa-miR-193a-5p+hsa-miR-193b-5p	1.31
hsa-miR-3065-3p	1.31
hsa-miR-3182	1.31
hsa-miR-410-3p	1.31
hsa-miR-450b-3p	1.31
hsa-miR-522-3p	1.31

hsa-miR-1-5p	1.3
hsa-miR-298	1.3
hsa-miR-328-3p	1.3
hsa-miR-3614-5p	1.3
hsa-miR-374b-5p	1.3
hsa-miR-4458	1.3
hsa-miR-499b-3p	1.3
hsa-miR-543	1.3
hsa-miR-595	1.3
hsa-miR-639	1.3
hsa-miR-1255a	1.29
hsa-miR-133a-3p	1.29
hsa-miR-142-5p	1.29
hsa-miR-212-3p	1.29
hsa-miR-222-3p	1.29
hsa-miR-3918	1.29
hsa-miR-454-3p	1.29
hsa-miR-504-3p	1.29
hsa-miR-520h	1.29
hsa-miR-575	1.29
hsa-miR-770-5p	1.29
hsa-miR-924	1.29
hsa-miR-1234-3p	1.28

hsa-miR-548e-3p	1.28
hsa-miR-300	1.27
hsa-miR-3605-3p	1.27
hsa-miR-432-5p	1.27
hsa-miR-519b-5p+hsa-miR-519c-5p+hsa-miR-523-5p+hsa-miR-518e-5p+hsa-miR-522-5p+hsa-miR-519a-5p	1.27
hsa-miR-582-3p	1.27
hsa-miR-1304-5p	1.26
hsa-miR-134-5p+hsa-miR-6728-5p	1.26
hsa-miR-216a-5p	1.26
hsa-miR-22-3p	1.26
hsa-miR-328-5p	1.26
hsa-miR-34c-5p	1.26
hsa-miR-433-3p	1.26
hsa-miR-491-3p	1.26
hsa-miR-10b-5p	1.25
hsa-miR-24-3p	1.25
hsa-miR-3130-3p	1.25
hsa-miR-4524a-5p	1.25
hsa-miR-487b-5p	1.25
hsa-miR-494-5p	1.25
hsa-miR-526b-5p	1.25
hsa-miR-572	1.25
hsa-miR-620	1.25

hsa-miR-654-5p	1.25
hsa-miR-125a-3p	1.24
hsa-miR-135b-5p	1.24
hsa-miR-137	1.24
hsa-miR-224-5p	1.24
hsa-miR-34b-3p	1.24
hsa-miR-516a-5p	1.24
hsa-miR-767-3p	1.24
hsa-miR-890	1.24
hsa-miR-1285-5p	1.23
hsa-miR-130a-3p	1.23
hsa-miR-139-5p	1.23
hsa-miR-320b	1.23
hsa-miR-4435	1.23
hsa-miR-499a-5p	1.23
hsa-miR-146a-5p	1.22
hsa-miR-3192-5p	1.22
hsa-miR-3614-3p	1.22
hsa-miR-4448	1.22
hsa-miR-548ah-5p	1.22
hsa-miR-885-5p	1.22
hsa-miR-936	1.22
hsa-miR-125b-5p	1.21

hsa-miR-210-3p	1.21
hsa-miR-23b-3p	1.21
hsa-miR-377-3p	1.21
hsa-miR-99b-5p	1.21
hsa-miR-100-5p	1.2
hsa-miR-1204	1.2
hsa-miR-1303	1.2
hsa-miR-181a-3p	1.2
hsa-miR-187-3p	1.2
hsa-miR-376a-3p	1.2
hsa-miR-421	1.2
hsa-miR-450b-5p	1.2
hsa-miR-4787-3p	1.2
hsa-miR-493-3p	1.2
hsa-let-7f-5p	1.19
hsa-miR-1224-3p	1.19
hsa-miR-1257	1.19
hsa-miR-1269a	1.19
hsa-miR-1295a	1.19
hsa-miR-142-3p	1.19
hsa-miR-485-3p	1.19
hsa-miR-488-3p	1.19
hsa-miR-1307-5p	1.18

hsa-miR-151a-3p	1.18
hsa-miR-4707-5p	1.18
hsa-miR-495-3p	1.18
hsa-miR-5010-5p	1.18
hsa-miR-520a-3p	1.18
hsa-miR-579-5p	1.18
hsa-miR-1269b	1.17
hsa-miR-501-3p	1.17
hsa-miR-517a-3p	1.17
hsa-miR-521	1.17
hsa-miR-548b-3p	1.17
hsa-miR-101-3p	1.16
hsa-miR-1296-5p	1.16
hsa-miR-181b-5p+hsa-miR-181d-5p	1.16
hsa-miR-190a-3p	1.16
hsa-miR-129-2-3p	1.15
hsa-miR-1323	1.15
hsa-miR-3151-5p	1.15
hsa-miR-548g-3p	1.15
hsa-miR-576-5p	1.15
hsa-miR-1827	1.14
hsa-miR-491-5p	1.14
hsa-miR-941	1.14

hsa-miR-1233-3p	1.13
hsa-miR-127-3p	1.13
hsa-miR-199a-5p	1.13
hsa-miR-2053	1.13
hsa-miR-507	1.13
hsa-miR-525-3p	1.13
hsa-miR-548o-3p+hsa-miR-548ah-3p+hsa-miR-548av-3p	1.13
hsa-miR-548y	1.13
hsa-miR-655-3p	1.13
hsa-miR-874-3p	1.13
hsa-miR-509-3p	1.12
hsa-miR-564	1.12
hsa-miR-617	1.12
hsa-miR-92b-3p	1.12
hsa-miR-1-3p	1.11
hsa-miR-27b-3p	1.11
hsa-miR-548n	1.11
hsa-miR-578	1.11
hsa-miR-584-3p	1.11
hsa-miR-769-5p	1.11
hsa-miR-1248	1.1
hsa-miR-1976	1.1
hsa-miR-362-5p	1.1

hsa-miR-455-3p	1.1
hsa-miR-515-3p	1.1
hsa-miR-106b-5p	1.09
hsa-miR-138-5p	1.09
hsa-miR-188-3p	1.09
hsa-miR-34a-5p	1.09
hsa-miR-591	1.09
hsa-miR-651-3p	1.09
hsa-miR-1273c	1.08
hsa-miR-1908-3p	1.08
hsa-miR-198	1.08
hsa-miR-297	1.08
hsa-miR-30d-5p	1.08
hsa-miR-450a-5p	1.08
hsa-miR-630	1.08
hsa-miR-652-5p	1.08
hsa-miR-1307-3p	1.07
hsa-miR-376b-3p	1.07
hsa-miR-450a-1-3p	1.07
hsa-miR-5196-5p	1.07
hsa-miR-545-3p	1.07
hsa-miR-548ar-3p	1.07
hsa-miR-761	1.07

hsa-miR-766-3p	1.07
hsa-miR-98-3p	1.07
hsa-miR-194-5p	1.06
hsa-miR-514a-5p	1.06
hsa-miR-548d-3p	1.06
hsa-miR-573	1.06
hsa-miR-876-5p	1.06
hsa-miR-95-3p	1.06
hsa-miR-1250-5p	1.05
hsa-miR-203a-3p	1.05
hsa-miR-33a-5p	1.05
hsa-miR-499a-3p	1.05
hsa-miR-1972	1.04
hsa-miR-371a-5p	1.04
hsa-miR-375	1.04
hsa-miR-431-5p	1.04
hsa-miR-4792	1.04
hsa-miR-642a-5p	1.04
hsa-let-7e-5p	1.03
hsa-miR-133b	1.03
hsa-miR-30e-3p	1.03
hsa-miR-378d	1.03
hsa-miR-492	1.03

hsa-miR-671-3p	1.03
hsa-miR-502-3p	1.02
hsa-miR-888-5p	1.02
hsa-miR-1271-5p	1.01
hsa-miR-1322	1.01
hsa-miR-148b-3p	1.01
hsa-miR-596	1.01
hsa-miR-661	1
hsa-miR-1298-5p	-1
hsa-miR-326	-1.01
hsa-miR-4284	-1.01
hsa-miR-451a	-1.01
hsa-miR-517b-3p	-1.01
hsa-miR-600	-1.01
hsa-miR-26a-5p	-1.02
hsa-miR-499b-5p	-1.02
hsa-miR-548ak	-1.02
hsa-miR-129-5p	-1.03
hsa-miR-448	-1.03
hsa-miR-4707-3p	-1.03
hsa-miR-486-3p	-1.03
hsa-miR-514b-5p	-1.03
hsa-miR-5196-3p+hsa-miR-6732-3p	-1.03

hsa-miR-199b-5p	-1.04
hsa-miR-361-5p	-1.04
hsa-miR-376c-3p	-1.04
hsa-miR-607	-1.04
hsa-miR-107	-1.05
hsa-miR-190b	-1.05
hsa-miR-320e	-1.05
hsa-miR-2278	-1.06
hsa-miR-520f-3p	-1.06
hsa-miR-642a-3p	-1.06
hsa-miR-1276	-1.07
hsa-miR-147a	-1.07
hsa-miR-15a-5p	-1.07
hsa-miR-3136-5p	-1.07
hsa-miR-656-3p	-1.07
hsa-miR-98-5p	-1.07
hsa-miR-1255b-5p	-1.08
hsa-miR-30a-5p	-1.08
hsa-miR-323a-5p	-1.08
hsa-miR-6724-5p	-1.08
hsa-miR-515-5p	-1.09
hsa-miR-606	-1.1
hsa-miR-802	-1.1

hsa-miR-644a	-1.11
hsa-miR-660-3p	-1.11
hsa-miR-188-5p	-1.12
hsa-miR-610	-1.12
hsa-miR-513c-3p	-1.13
hsa-miR-135a-5p	-1.14
hsa-miR-378h	-1.14
hsa-miR-301a-3p	-1.15
hsa-miR-604	-1.15
hsa-miR-106a-5p+hsa-miR-17-5p	-1.16
hsa-miR-1203	-1.16
hsa-miR-125a-5p	-1.16
hsa-miR-324-5p	-1.16
hsa-miR-875-3p	-1.17
hsa-miR-16-5p	-1.19
hsa-miR-4521	-1.19
hsa-miR-342-3p	-1.2
hsa-miR-574-5p	-1.2
hsa-miR-378i	-1.22
hsa-miR-423-3p	-1.22
hsa-miR-874-5p	-1.23
hsa-miR-1260a	-1.24
hsa-miR-30c-5p	-1.24

hsa-miR-520a-5p	-1.26
hsa-miR-935	-1.3
hsa-miR-562	-1.32
hsa-miR-9-5p	-1.32
hsa-miR-128-1-5p	-1.33
hsa-miR-361-3p	-1.33
hsa-miR-21-5p	-1.34
hsa-miR-1206	-1.35
hsa-miR-130b-3p	-1.36
hsa-miR-1247-5p	-1.37
hsa-miR-548d-5p	-1.37
hsa-miR-574-3p	-1.38
hsa-miR-5001-3p	-1.39
hsa-miR-208a-3p	-1.42
hsa-miR-877-5p	-1.43
hsa-miR-197-3p	-1.44
hsa-miR-940	-1.46
hsa-miR-221-3p	-1.48
hsa-miR-20a-5p+hsa-miR-20b-5p	-1.52
hsa-miR-3158-3p	-1.52
hsa-miR-520d-5p+hsa-miR-527+hsa-miR-518a-5p	-1.52
hsa-miR-1908-5p	-1.58
hsa-miR-4454+hsa-miR-7975	-1.58

hsa-miR-25-3p	-1.59
hsa-miR-3916	-1.61
hsa-miR-4443	-1.66
hsa-miR-155-5p	-1.7
ACTB	-1.7
hsa-miR-423-5p	-2.36
hsa-miR-181a-5p	-2.39
B2M	-2.78
hsa-miR-93-5p	-2.84
hsa-let-7i-5p	-2.9
hsa-miR-92a-3p	-2.95
hsa-miR-223-3p	-3.13
hsa-let-7g-5p	-3.18
hsa-miR-23a-3p	-3.41
hsa-let-7d-5p	-5.33
hsa-miR-3065-5p	-6.02
hsa-let-7c-5p	-6.14
hsa-miR-15b-5p	-6.27
hsa-miR-191-5p	-10.17
hsa-let-7b-5p	-11.63
hsa-let-7a-5p	-340.82

References

1. Billingham RE, Brent L, Medawar PB. “Actively acquired tolerance” of foreign cells. 1953. *J. Immunol.* 2010;184(1):5–8.

2. BILLINGHAM RE, BRENT L, MEDAWAR PB. The antigenic stimulus in transplantation immunity. *Nature*. 1956;178(4532):514–519.

3. BURNET M. Cancer; a biological approach. I. The processes of control. *Br. Med. J.* 1957;1(5022):779–786.

4. Thomas. In Cellular and Humoral Aspects of the Hypersensitive States. Hoeber-Harper; 1959.

5. Birkeland SA, Storm HH, Lamm LU, et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. *Int. J. Cancer*. 1995;60(2):183–189.

6. Butturini A, Bortin MM, Gale RP. Graft-versus-leukemia following bone marrow transplantation. *Bone Marrow Transplant.* 1987;2(3):233–242.

7. Hassane M. Zarour, MD, Albert DeLeo, PhD, Olivera J. Finn, PhD, and Walter J. Storkus P. Cancer Medicine.

8. Nath S, Mukherjee P. MUC1: A multifaceted oncoprotein with a key role in cancer progression. *Trends Mol. Med.* 2014;20(6):332–342.

9. Ascierto ML, Melero I, Ascierto PA. Melanoma: From Incurable Beast to a Curable Bet. The Success of Immunotherapy. *Front. Oncol.* 2015;5:152.

10. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 2004;22:329–360.

11. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. 2007;450(7171):903–907.

12. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of

"tumor escape" phenotypes. *Nat. Immunol.* 2002;3(11):999–1005.

13. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*. 1992;71(7):1093–1102.

14. Speiser DE, Miranda R, Zakarian A, et al. Self antigens expressed by solid tumors Do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J. Exp. Med.* 1997;186(5):645–653.

15. Gerlini G, Tun-Kyi A, Dudli C, et al. Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am. J. Pathol.* 2004;165(6):1853–1863.

16. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* 1996;2(10):1096–1103.

17. Ma Y, Shurin G V., Peiyuan Z, Shurin MR. Dendritic cells in the cancer microenvironment. *J. Cancer*. 2013;4(1):36–44.

18. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 2003;21:685–711.

19. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. *Clin. Cancer Res.* 2000;6(5):1755–1766.

20. Nestle FO, Burg G, Fäh J, Wrone-Smith T, Nickoloff BJ. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am. J. Pathol.* 1997;150(2):641–651.

21. Troy A, Davidson P, Atkinson C, Hart D. Phenotypic characterisation of the dendritic cell infiltrate in prostate cancer. *J. Urol.* 1998;160(1):214–219.

22. Hartmann E, Wollenberg B, Rothenfusser S, et al. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res.* 2003;63(19):6478–6487.

23. Facchetti F, Vermi W, Mason D, Colonna M. The plasmacytoid monocyte/interferon producing cells. *Virchows Arch.* 2003;443(6):703–717.

24. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat. Med.* 2001;7(12):1339–1346.

25. Liyanage UK, Moore TT, Joo H-G, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol.* 2002;169(5):2756–2761.

26. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 2004;10(9):942–949.

27. Halvorsen EC, Mahmoud SM, Bennewith KL. Emerging roles of regulatory T cells in tumour progression and metastasis. *Cancer Metastasis Rev.* 2014;33(4):1025–41.

28. Berger CL, Tigelaar R, Cohen J, et al. Cutaneous T-cell lymphoma: Malignant proliferation of T-regulatory cells. *Blood*. 2005;105(4):1640–1647.

29. Beyer M, Kochanek M, Darabi K, et al. Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood*. 2005;106(6):2018–2025.

30. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 2012;12(4):253–268.

31. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 2002;8(8):793–

800.

32. Malmberg KJ, Levitsky V, Norell H, et al. IFN-γ protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J. Clin. Invest.* 2002;110(10):1515–1523.

33. Derré L, Corvaisier M, Charreau B, et al. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J. Immunol.* 2006;177(5):3100–3107.

34. Srivastava N, Avigan D, Rosenblatt J. PD-1 blockade in cancer immunotherapy. *Drugs Future*. 2014;39(2):129–132.

35. Weber J. Immune checkpoint proteins: A new therapeutic paradigm for cancerpreclinical background: CTLA-4 and PD-1 blockade. *Semin. Oncol.* 2010;37(5):430–439.

36. Borch TH, Donia M, Andersen MH, Svane IM. Reorienting the immune system in the treatment of cancer by using anti-PD-1 and anti-PD-L1 antibodies. *Drug Discov. Today*. 2015;

37. Munn DH, Shafizadeh E, Attwood JT, et al. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* 1999;189(9):1363–1372.

38. Zamarron BF, Chen W. Dual roles of immune cells and their factors in cancer development and progression. *Int. J. Biol. Sci.* 2011;7(5):651–658.

39. Society AC. Leukemia AML Guide.

40. Falini B, Tiacci E, Martelli MP, Ascani S, Pileri SA. New classification of acute myeloid leukemia and precursor-related neoplasms: changes and unsolved issues. *Discov. Med.* 2010;10(53):281–292.

41. Vardiman J, Reichard K. Acute Myeloid Leukemia With Myelodysplasia-Related Changes. *Am. J. Clin. Pathol.* 2015;144(1):29–43.

42. Feldman EJ. Novel Therapeutics for Therapy-Related Acute Myeloid Leukemia: 2014. *Clin. Lymphoma. Myeloma Leuk.* 2015;15 Suppl:S91-3.

43. Bueso-Ramos CE, Kanagal-Shamanna R, Routbort MJ, Hanson CA. Therapy-Related Myeloid Neoplasms. *Am. J. Clin. Pathol.* 2015;144(2):207–18.

44. Khalade A, Jaakkola MS, Pukkala E, Jaakkola JJK. Exposure to benzene at work and the risk of leukemia: a systematic review and meta-analysis. *Environ. Health*. 2010;9:31.

45. Caldwell JT, Ge Y, Taub JW. Prognosis and management of acute myeloid leukemia in patients with Down syndrome. *Expert Rev. Hematol.* 2014;7(6):831–40.

46. Alter BP. Fanconi anemia and the development of leukemia. *Best Pract. Res. Clin. Haematol.* 27(3–4):214–21.

47. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br. J. Haematol.* 1976;33(4):451–458.

48. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann. Intern. Med.* 1985;103(4):620–625.

49. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. 1998.

50. Ossenkoppele G, Löwenberg B. How I treat the older patient with acute myeloid leukemia. *Blood*. 2015;125(5):767–74.

51. Yates JW, Wallace HJ, Ellison RR, Holland JF. Cytosine arabinoside (NSC-63878)

and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia.

52. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect.* 62:21–78.

53. Cornelissen JJ, Van Putten WLJ, Verdonck LF, et al. Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: Benefits for whom? *Blood*. 2007;109(9):3658–3666.

54. Cornelissen JJ, Gratwohl A, Schlenk RF, et al. The European LeukemiaNet AML Working Party consensus statement on allogeneic HSCT for patients with AML in remission: an integrated-risk adapted approach. *Nat. Rev. Clin. Oncol.* 2012;9(10):579–590.

55. Gratwohl A, Baldomero H, Horisberger B, et al. Current trends in hematopoietic stem cell transplantation in Europe. *Blood*. 2002;100(7):2374–2386.

56. Kurosawa S, Yamaguchi T, Uchida N, et al. Comparison of allogeneic hematopoietic cell transplantation and chemotherapy in elderly patients with non-M3 acute myelogenous leukemia in first complete remission. *Biol. Blood Marrow Transplant.* 2011;17(3):401–411.

57. Tsimberidou A-M, Stavroyianni N, Viniou N, et al. Comparison of allogeneic stem cell transplantation, high-dose cytarabine, and autologous peripheral stem cell transplantation as postremission treatment in patients with de novo acute myelogenous leukemia. 2003.

58. Büchner T, Berdel WE, Schoch C, et al. Double induction containing either two courses or one course of high-dose cytarabine plus mitoxantrone and postremission therapy by either autologous stem-cell transplantation or by prolonged maintenance for acute myeloid leukemia. *J. Clin. Oncol.* 2006;24(16):2480–2489.

59. Rowe JM, Löwenberg B. Gemtuzumab ozogamicin in acute myeloid leukemia: a

remarkable saga about an active drug. *Blood*. 2013;121(24):4838–4841.

60. Kim TK, Gore SD, Zeidan AM. Epigenetic Therapy in Acute Myeloid Leukemia: Current and Future Directions. *Semin. Hematol.* 2015;52(3):172–83.

61. Dombret H, Seymour J, Butrym A, et al. International phase 3 study of azacitidine vs conventional care regimens in older patients with newly diagnosed AML with >30% blasts. *Blood*. 2015;

62. Kantarjian HM, Thomas XG, Dmoszynska A, et al. Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. *J. Clin. Oncol.* 2012;30(21):2670–2677.

63. H.M. Kantarjian, E. Jabbour, K. Yee et al. No Title. *Am. Soc. Hematol. Annu. Meet. Expo.* 2013;

64. Kuendgen A, Knipp S, Fox F, et al. Results of a phase 2 study of valproic acid alone or in combination with all-trans retinoic acid in 75 patients with myelodysplastic syndrome and relapsed or refractory acute myeloid leukemia. 2005.

65. Garcia-Manero G, Yang H, Bueso-Ramos C, et al. Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes. *Blood*. 2008;111(3):1060–1066.

66. Zeidner JF, Foster MC, Blackford AL, et al. Randomized multicenter phase 2 study of flavopiridol (alvocidib), cytarabine, and mitoxantrone (FLAM) versus cytarabine/daunorubicin (7+3) in newly diagnosed acute myeloid leukemia. *Haematologica*. 2015;

67. Levis M, Ravandi F, Wang ES, et al. Results from a randomized trial of salvage chemotherapy followed by lestaurtinib for patients with FLT3 mutant AML in first

relapse. *Blood*. 2011;117(12):3294–3301.

68. Borthakur G, Kantarjian H, Ravandi F, et al. Phase I study of sorafenib in patients with refractory or relapsed acute leukemias. *Haematologica*. 2011;96(1):62–68.

69. Metzelder S, Wang Y, Wollmer E, et al. Compassionate use of sorafenib in FLT3-ITD-positive acute myeloid leukemia: sustained regression before and after allogeneic stem cell transplantation. 2009.

70. Ravandi F, Alattar ML, Grunwald MR, et al. Phase 2 study of azacytidine plus sorafenib in patients with acute myeloid leukemia and FLT-3 internal tandem duplication mutation. *Blood*. 2013;121(23):4655–4662.

71. Tettamanti S, Marin V, Pizzitola I, et al. Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. *Br. J. Haematol.* 2013;161(3):389–401.

72. Konopleva M, Hogge DE, Rizzieri DA et al. No Title. 2012;

73. Marcucci G., Moser B., Blum W., Stock W., Wetzler M., Kolitz J.E., Thakuri M., Carter T., Stuart R.K. LRA. No Title. *ASCO*. 2007;

74. Moore J, Seiter K, Kolitz J, et al. A Phase II study of Bcl-2 antisense (oblimersen sodium) combined with gemtuzumab ozogamicin in older patients with acute myeloid leukemia in first relapse. *Leuk. Res.* 2006;30(7):777–783.

75. Stengel C, Jenner E, Meja K, Mayekar S, Khwaja A. Proliferation of PTEN-deficient haematopoietic tumour cells is not affected by isoform-selective inhibition of p110?? PI3-kinase and requires blockade of all class 1 PI3K activity. *Br. J. Haematol.* 2013;162(2):285–289.

76. Kampa-Schittenhelm KM, Heinrich MC, Akmut F, et al. Cell cycle-dependent activity of the novel dual PI3K-MTORC1/2 inhibitor NVP-BGT226 in acute leukemia. *Mol. Cancer*. 2013;12:46.

77. Altman JK, Sassano A, Kaur S, et al. Dual mTORC2/mTORC1 targeting results in potent suppressive effects on Acute Myeloid Leukemia (AML) progenitors. *Clin. Cancer Res.* 2011;17(13):4378–4388.

78. Zeng Z, Shi YX, Tsao T, et al. Targeting of mTORC1/2 by the mTOR kinase inhibitor PP242 induces apoptosis in AML cells under conditions mimicking the bone marrow microenvironment. *Blood*. 2012;120(13):2679–2689.

79. Reuter CW, Morgan MA, Bergmann L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? *Blood*. 2000;96(5):1655–1669.

80. Erba HP, Othus M, Walter RB, et al. Four different regimens of farnesyltransferase inhibitor tipifarnib in older, untreated acute myeloid leukemia patients: North American Intergroup Phase II study SWOG S0432. *Leuk. Res.* 2014;38(3):329–333.

81. Burnett AK, Russell NH, Culligan D, et al. The addition of the farnesyl transferase inhibitor, tipifarnib, to low dose cytarabine does not improve outcome for older patients with AML. *Br. J. Haematol.* 2012;158(4):519–522.

82. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res*. 1991;(262):3–11.

83. Fozza C, Bellizzi S, Bonfigli S, et al. Cytogenetic and hematological spontaneous remission in a case of acute myelogenous leukemia. *Eur. J. Haematol.* 2004;73(3):219–222.

84. Ifrah N, James JM, Viguie F, Marie JP, Zittoun R. Spontaneous remission in adult acute leukemia. *Cancer*. 1985;56(5):1187–1190.

85. Maywald O, Buchheidt D, Bergmann J, et al. Spontaneous remission in adult acute myeloid leukemia in association with systemic bacterial infection-case report and review of the literature. *Ann. Hematol.* 2004;83(3):189–194.

86. Trof RJ, Beishuizen A, Wondergem MJ, Strack van Schijndel RJM. Spontaneous remission of acute myeloid leukaemia after recovery from sepsis. *Neth. J. Med.* 2007;65(7):259–262.

87. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75(3):555–562.

88. Brouwer RE, Zwinderman KH, Kluin-Nelemans HC, et al. Expression and induction of costimulatory and adhesion molecules on acute myeloid leukemic cells: implications for adoptive immunotherapy. *Exp. Hematol.* 2000;28(2):161–168.

89. Vollmer M, Li L, Schmitt A, et al. Expression of human leucocyte antigens and co-stimulatory molecules on blasts of patients with acute myeloid leukaemia. *Br. J. Haematol.* 2003;120(6):1000–1008.

90. Whiteway A, Corbett T, Anderson R, Macdonald I, Grant Prentice H. Expression of co-stimulatory molecules on acute myeloid leukaemia blasts may effect duration of first remission. *Br. J. Haematol.* 2003;120(3):442–451.

91. Freeman-Keller M, Weber JS. Anti-programmed death receptor 1 immunotherapy in melanoma: rationale, evidence and clinical potential. *Ther Adv Med Oncol*. 2015;7(1):12–21.

92. Napolitano M, D'Alterio C, Cardone E, et al. Peripheral myeloid-derived suppressor and T regulatory PD-1 positive cells predict response to neoadjuvant short-course radiotherapy in rectal cancer patients. *Oncotarget*. 2015;6(10):8261–70.

93. Noman MZ, Desantis G, Janji B, et al. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J. Exp. Med.* 2014;211(5):781–90.

94. Kong Y, Zhang J, Claxton DF, et al. PD-1(hi)TIM-3(+) T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell

transplantation. *Blood Cancer J.* 2015;5:e330.

95. Berthon C, Driss V, Liu J, et al. In acute myeloid leukemia, B7-H1 (PD-L1) protection of blasts from cytotoxic T cells is induced by TLR ligands and interferon-gamma and can be reversed using MEK inhibitors. *Cancer Immunol. Immunother.* 2010;59(12):1839–1849.

96. Rosenblatt J, Glotzbecker B, Mills H, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J Immunother*. 2011;34(5):409–418.

97. Zhou Q, Munger ME, Veenstra RG, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood*. 2011;117(17):4501–4510.

98. Gao L, Yu S, Zhang X. Hypothesis: Tim-3/galectin-9, a new pathway for leukemia stem cells survival by promoting expansion of myeloid-derived suppressor cells and differentiating into tumor-associated macrophages. *Cell Biochem. Biophys.* 2014;70(1):273–7.

99. Kikushige Y, Miyamoto T, Yuda J, et al. A TIM-3/Gal-9 Autocrine Stimulatory Loop Drives Self-Renewal of Human Myeloid Leukemia Stem Cells and Leukemic Progression. *Cell Stem Cell*. 2015;

100. Folgiero V, Cifaldi L, Li Pira G, et al. TIM-3/Gal-9 interaction induces IFNγ-dependent IDO1 expression in acute myeloid leukemia blast cells. *J. Hematol. Oncol.* 2015;8:36.

101. Li C, Chen X, Yu X, et al. Tim-3 is highly expressed in T cells in acute myeloid leukemia and associated with clinicopathological prognostic stratification. *Int J Clin Exp Pathol*. 2014;7(10):6880–6888.

102. Laurent S, Palmisano GL, Martelli AM, et al. CTLA-4 expressed by chemoresistant, as well as untreated, myeloid leukaemia cells can be targeted with ligands to

induce apoptosis. *Br. J. Haematol.* 2007;136(4):597–608.

103. Zhong RK, Loken M, Lane TA, Ball ED. CTLA-4 blockade by a human MAb enhances the capacity of AML-derived DC to induce T-cell responses against AML cells in an autologous culture system. *Cytotherapy*. 2006;8(1):3–12.

104. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 2004;64(16):5839–5849.

105. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood*. 2007;109(4):1568–1573.

106. Oberlies J, Watzl C, Giese T, et al. Regulation of NK cell function by human granulocyte arginase. *J. Immunol.* 2009;182(9):5259–5267.

107. Mussai F, Egan S, Higginbotham-Jones J, et al. Arginine dependence of acute myeloid leukemia blast proliferation: a novel therapeutic target. *Blood*. 2015;125(15):2386–96.

108. Mussai F, De Santo C, Abu-Dayyeh I, et al. Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment. *Blood*. 2013;122(5):749–758.

109. Folgiero V, Goffredo BM, Filippini P, et al. Indoleamine 2,3-dioxygenase 1 (IDO1) activity in leukemia blasts correlates with poor outcome in childhood acute myeloid leukemia. *Oncotarget*. 2014;5(8):2052–64.

110. Curti A, Trabanelli S, Salvestrini V, Baccarani M, Lemoli RM. The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: Focus on hematology. *Blood*. 2009;113(11):2394–2401.

111. El Kholy NM, Sallam MM, Ahmed MB, et al. Expression of indoleamine 2,3-dioxygenase in acute myeloid leukemia and the effect of its inhibition on cultured

leukemia blast cells. *Med. Oncol.* 2011;28(1):270–278.

112. Chruscinski A, Sadozai H, Rojas-Luengas V, et al. Role of Regulatory T Cells (Treg) and the Treg Effector Molecule Fibrinogen-like Protein 2 in Alloimmunity and Autoimmunity. *Rambam Maimonides Med. J.* 2015;6(3):.

113. Schick J, Vogt V, Zerwes M, et al. Antileukemic T-cell responses can be predicted by the composition of specific regulatory T-cell subpopulations. *J. Immunother.* 2013;36(4):223–37.

114. Yang W, Xu Y. Clinical significance of Treg cell frequency in acute myeloid leukemia. *Int. J. Hematol.* 2013;98(5):558–562.

115. Shenghui Z, Yixiang H, Jianbo W, et al. Elevated frequencies of $CD4^+$ $CD25^+$ CD127lo regulatory T cells is associated to poor prognosis in patients with acute myeloid leukemia. *Int. J. Cancer*. 2011;129(6):1373–1381.

116. Rooney CM. Can Treg elimination enhance NK cell therapy for AML? *Blood*. 2014;123(25):3848–9.

117. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: New biology and insights into the graft-versus-leukemia effect. *Blood*. 2002;100(6):1935–1947.

118. Sanchez-Correa B, Campos C, Pera A, et al. Natural killer cell immunosenescence in acute myeloid leukaemia patients: new targets for immunotherapeutic strategies? *Cancer Immunol. Immunother.* 2015;

119. Lichtenegger FS, Lorenz R, Gellhaus K, et al. Impaired NK cells and increased T regulatory cell numbers during cytotoxic maintenance therapy in AML. *Leuk. Res.* 2014;38(8):964–9.

120. Stringaris K, Sekine T, Khoder A, et al. Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in

acute myeloid leukemia. *Haematologica*. 2014;99(5):836–847.

121. Lowdell MW, Craston R, Samuel D, et al. Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells. *Br. J. Haematol.* 2002;117(4):821–827.

122. Lissoni P, Vigore L, Ferranti R, et al. Circulating dendritic cells in early and advanced cancer patients: diminished percent in the metastatic disease. *J Biol Regul Homeost Agents*. 1999;13(4):216–219.

123. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol*. 2004;4(12):941–952.

124. Demoulin S, Herfs M, Delvenne P, Hubert P. Tumor microenvironment converts plasmacytoid dendritic cells into immunosuppressive/tolerogenic cells: insight into the molecular mechanisms. *J Leukoc Biol*. 2013;93(3):343–352.

125. Mohty M, Jarrossay D, Lafage-Pochitaloff M, et al. Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. *Blood*. 2001;98(13):3750–3756.

126. Rosenblatt J, Avivi I, Vasir B, et al. Vaccination with dendritic cell/tumor fusions following autologous stem cell transplant induces immunologic and clinical responses in multiple myeloma patients. *Clin Cancer Res*. 2013;19(13):3640–3648.

127. Luptakova K, Rosenblatt J, Glotzbecker B, et al. Lenalidomide enhances anti-myeloma cellular immunity. *Cancer Immunol Immunother*. 2013;62(1):39–49.

128. Stroopinsky D, Rosenblatt J, Ito K, et al. MUC1 is a potential target for the treatment of acute myeloid leukemia stem cells. *Cancer Res*. 2013;73(17):5569–5579.

129. Liu S, Yin L, Stroopinsky D, et al. MUC1-C oncoprotein promotes FLT3 receptor activation in acute myeloid leukemia cells. *Blood*. 2014;123(5):734–742.

130. Jain S, Stroopinsky D, Yin L, et al. Mucin 1 is a potential therapeutic target in cutaneous T-cell lymphoma. *Blood*. 2015;126(3):354–62.

131. Raina D, Agarwal P, Lee J, et al. Characterization of the MUC1-C Cytoplasmic Domain as a Cancer Target. *PLoS One*. 2015;10(8):e0135156.

132. Yin L, Ahmad R, Kosugi M, et al. Survival of human multiple myeloma cells is dependent on MUC1 C-terminal transmembrane subunit oncoprotein function. *Mol. Pharmacol.* 2010;78(2):166–174.

133. Nencioni A, Grünebach F, Schmidt SM, et al. The use of dendritic cells in cancer immunotherapy. *Crit Rev Oncol Hematol*. 2008;65(3):191–199.

134. Avigan D. Dendritic cells: development, function and potential use for cancer immunotherapy. *Blood Rev*. 1999;13(1):51–64.

135. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271–296.

136. Dubois B, Massacrier C, Caux C. Selective attraction of naive and memory B cells by dendritic cells. *J Leukoc Biol*. 2001;70(4):633–641.

137. Jonuleit H, Kühn U, Müller G, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol*. 1997;27(12):3135–3142.

138. Wimmers F, Schreibelt G, Sköld AE, Figdor CG, De Vries IJ. Paradigm Shift in Dendritic Cell-Based Immunotherapy: From in vitro Generated Monocyte-Derived DCs to Naturally Circulating DC Subsets. *Front Immunol*. 2014;5:165.

139. Märten A, Renoth S, Heinicke T, et al. Allogeneic dendritic cells fused with tumor cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma. *Hum Gene Ther*. 2003;14(5):483–494.

140. Avigan DE Kantoff PW GDJ. Interim safety and efficacy results from a phase I/II

study of vaccination with electrofused allogeneic dendritic cells/autologous tumor-derived cells in patients with stage IV renal cell carcinoma. 2004;

141. Hus I, Roliński J, Tabarkiewicz J, et al. Allogeneic dendritic cells pulsed with tumor lysates or apoptotic bodies as immunotherapy for patients with early-stage B-cell chronic lymphocytic leukemia. *Leukemia*. 2005;19(9):1621–1627.

142. Philip R, Alters SE, Brunette E, et al. Dendritic cells loaded with MART-1 peptide or infected with adenoviral construct are functionally equivalent in the induction of tumor-specific cytotoxic T lymphocyte responses in patients with melanoma. *J Immunother*. 2000;23(1):168–176.

143. Minev BR, Chavez FL, Dudouet BM, Mitchell MS. Synthetic insertion signal sequences enhance MHC class I presentation of a peptide from the melanoma antigen MART-1. *Eur J Immunol*. 2000;30(8):2115–2124.

144. Cao J, Jin Y, Li W, et al. DNA vaccines targeting the encoded antigens to dendritic cells induce potent antitumor immunity in mice. *BMC Immunol*. 2013;14:39.

145. Li D, Hua S, Fan Y, et al. DNA vaccine expressing repeated carcinoembryonic antigen (CEA)(625-667) induces strong immunity in mice. *Immunol Lett*. 2011;135(1–2):124–128.

146. Signori E, Iurescia S, Massi E, et al. DNA vaccination strategies for anti-tumour effective gene therapy protocols. *Cancer Immunol Immunother*. 2010;59(10):1583–1591.

147. Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J Exp Med*. 1996;184(2):465–472.

148. Nair SK, Morse M, Boczkowski D, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. *Ann Surg*. 2002;235(4):540–549.

149. Hamilton DH, Litzinger MT, Jales A, et al. Immunological targeting of tumor cells undergoing an epithelial-mesenchymal transition via a recombinant brachyury-yeast vaccine. *Oncotarget*. 2013;

150. Litzinger MT, Foon KA, Sabzevari H, et al. Chronic lymphocytic leukemia (CLL) cells genetically modified to express B7-1, ICAM-1, and LFA-3 confer APC capacity to T cells from CLL patients. *Cancer Immunol Immunother*. 2009;58(6):955–965.

151. Litzinger MT, Foon KA, Tsang KY, Schlom J, Palena C. Comparative analysis of MVA-CD40L and MVA-TRICOM vectors for enhancing the immunogenicity of chronic lymphocytic leukemia (CLL) cells. *Leuk Res*. 2010;34(10):1351–1357.

152. Palma M, Hansson L, Choudhury A, et al. Vaccination with dendritic cells loaded with tumor apoptotic bodies (Apo-DC) in patients with chronic lymphocytic leukemia: effects of various adjuvants and definition of immune response criteria. *Cancer Immunol Immunother*. 2012;61(6):865–879.

153. Avigan D, Rosenblatt J, Kufe D. Dendritic/tumor fusion cells as cancer vaccines. *Semin Oncol*. 2012;39(3):287–295.

154. Koido S, Homma S, Okamoto M, et al. Fusions between dendritic cells and whole tumor cells as anticancer vaccines. *Oncoimmunology*. 2013;2(5):e24437.

155. Amos SM, Duong CP, Westwood JA, et al. Autoimmunity associated with immunotherapy of cancer. *Blood*. 2011;118(3):499–509.

156. Rosenblatt J, Vasir B, Uhl L, et al. Vaccination with dendritic cell/tumor fusion cells results in cellular and humoral antitumor immune responses in patients with multiple myeloma. *Blood*. 2011;117(2):393–402.

157. Reichardt VL, Okada CY, Liso A, et al. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma--a feasibility study. *Blood*. 1999;93(7):2411–2419.

158. Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood*. 2002;99(5):1517–1526.

159. Schuster SJ. Idiotype vaccine therapy (BiovaxID) in follicular lymphoma in first complete remission: Phase III clinical trial results. *Univ. Pennsylvania*. 2009;

160. Lacy MQ, Mandrekar S, Dispenzieri A, et al. Idiotype-pulsed antigen-presenting cells following autologous transplantation for multiple myeloma may be associated with prolonged survival. *Am J Hematol*. 2009;84(12):799–802.

161. Röllig C, Schmidt C, Bornhäuser M, et al. Induction of cellular immune responses in patients with stage-I multiple myeloma after vaccination with autologous idiotype-pulsed dendritic cells. *J Immunother*. 2011;34(1):100–106.

162. Van Tendeloo VF, Van de Velde A, Van Driessche A, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. *Proc Natl Acad Sci U S A*. 2010;107(31):13824–13829.

163. Kitawaki T, Kadowaki N, Fukunaga K, et al. Cross-priming of CD8(+) T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia. *Exp Hematol*. 2011;39(4):424–433.e2.

164. Rosenblatt J, Stone R, Uhl L, et al. Clinical trial evaluating DC/AML fusion cell vaccination in AML patients who achieve a chemotherapy-induced remission. *ASH Annu. Meet.* 2013;

165. Houtenbos I, Westers TM, Hess CJ, et al. Flt-3 internal tandem duplication hampers differentiation of AML blasts towards leukemic dendritic cells. *Leukemia*. 2006;20(10):1892–1895.

166. Anguille S, Willemen Y, Lion E, Smits EL, Berneman ZN. Dendritic cell vaccination

in acute myeloid leukemia. *Cytotherapy*. 2012;14(6):647–656.

167. Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology*. 2013;138(2):105–115.

168. Rosenblatt J Vasir B, Uhl L, Katz T, Avigan D et al. AI. Blockade of PD-1 in Combination with Dendritic Cell/Myeloma Fusion Cell Vaccination Following Autologous Stem Cell Transplantation. *ASH 2012*. 2012;(Abstract 578):

169. Borrello IM, Levitsky HI, Stock W, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cellular immunotherapy in combination with autologous stem cell transplantation (ASCT) as postremission therapy for acute myeloid leukemia (AML). *Blood*. 2009;114(9):1736–1745.

170. Brune M, Castaigne S, Catalano J, et al. Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: Results of a randomized phase 3 trial. *Blood*. 2006;108(1):88–96.

171. Lichtenegger FS, Krupka C, Köhnke T, Subklewe M. Immunotherapy for Acute Myeloid Leukemia. *Semin. Hematol.* 2015;52(3):207–14.

172. Topp MS, Gökbuget N, Zugmaier G, et al. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. *Blood*. 2012;120(26):5185–5187.

173. Hussaini A.L., Ritchey J. RMP. Targeting CD123 in leukemic stem cells using dual affinity re-targeting molecules (DARTs). *ASH*. 2013;

174. Kochenderfer JN, Dudley ME, Kassim SH, et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *J. Clin. Oncol.* 2015;33(6):540–9.

175. Mauude SL. Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia. 2014;

176. Marin V, Pizzitola I, Agostoni V, et al. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: Improvement of their immune activity by expression of CD33-specific chimeric receptors. *Haematologica*. 2010;95(12):2144–2152.

177. Dutour A, Marin V, Pizzitola I, et al. In Vitro and In Vivo Antitumor Effect of Anti-CD33 Chimeric Receptor-Expressing EBV-CTL against CD33+ Acute Myeloid Leukemia. *Adv. Hematol.* 2012;2012:1–10.

178. Wang Q, Wang Y, Lv H, et al. Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia. *Mol. Ther.* 2015;23(1):184–91.

179. Mardiros A, Dos Santos C, McDonald T, et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood*. 2013;122(18):3138–3148.

180. Duwe AK, Singhal SK. The immunoregulatory role of bone marrow. I. Suppression of the induction of antibody responses to T-dependent and T-independent antigens by cells in the bone marrow. *Cell. Immunol.* 1979;43(2):362–371.

181. Bronte V, Wang M, Overwijk WW, et al. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J. Immunol.* 1998;161(10):5313–5320.

182. Kusmartsev SA, Li Y, Chen SH. Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J. Immunol.* 2000;165(2):779–785.

183. Gabrilovich D, Ishida T, Oyama T, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of

multiple hematopoietic lineages in vivo. *Blood*. 1998;92(11):4150–4166.

184. Watson GA, Fu YX, Lopez DM. Splenic macrophages from tumor-bearing mice co-expressing MAC-1 and MAC-2 antigens exert immunoregulatory functions via two distinct mechanisms. *J. Leukoc. Biol.* 1991;49(2):126–138.

185. Mazzoni A, Bronte V, Visintin A, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J. Immunol.* 2002;168(2):689–695.

186. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J. Immunol.* 2004;172(2):989–999.

187. Bronte V, Serafini P, De Santo C, et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J. Immunol.* 2003;170(1):270–278.

188. Bronte V, Apolloni E, Cabrelle A, et al. Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. *Blood*. 2000;96(12):3838–3846.

189. Mielcarek M, Martin PJ, Torok-Storb B. Suppression of alloantigen-induced T-cell proliferation by CD14+ cells derived from granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells. *Blood*. 1997;89(5):1629–1634.

190. Singh RK, Varney ML, Buyukberber S, et al. Fas-FasL-mediated CD4+ T-cell apoptosis following stem cell transplantation. *Cancer Res.* 1999;59(13):3107–3111.

191. Laoui D, Van Overmeire E, Movahedi K, et al. Mononuclear phagocyte heterogeneity in cancer: Different subsets and activation states reaching out at the tumor site. *Immunobiology*. 2011;216(11):1192–1202.

192. Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S SH. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.*

2007;67(1):425.

193. Mucha J, Majchrzak K, Taciak B, Hellmén E, Król M. MDSCs mediate angiogenesis and predispose canine mammary tumor cells for metastasis via IL-28/IL-28RA (IFN-λ) signaling. *PLoS One*. 2014;9(7):e103249.

194. Meirow Y, Kanterman J, Baniyash M. Paving the Road to Tumor Development and Spreading: Myeloid-Derived Suppressor Cells are Ruling the Fate. *Front. Immunol.* 2015;6:523.

195. Zhang G, Huang H, Zhu Y, et al. A novel subset of B7-H3(+)CD14(+)HLA-DR(-/low) myeloid-derived suppressor cells are associated with progression of human NSCLC. *Oncoimmunology*. 2015;4(2):e977164.

196. Li Z-L, Ye S-B, OuYang L-Y, et al. COX-2 promotes metastasis in nasopharyngeal carcinoma by mediating interactions between cancer cells and myeloid-derived suppressor cells. *Oncoimmunology*. 2015;4(11):e1044712.

197. Huang H, Zhang G, Li G, Ma H, Zhang X. Circulating CD14(+)HLA-DR(-/low) myeloid-derived suppressor cell is an indicator of poor prognosis in patients with ESCC. *Tumour Biol.* 2015;

198. Jeanbart L, Kourtis IC, van der Vlies AJ, Swartz MA, Hubbell JA. 6-Thioguanine-loaded polymeric micelles deplete myeloid-derived suppressor cells and enhance the efficacy of T cell immunotherapy in tumor-bearing mice. *Cancer Immunol. Immunother.* 2015;64(8):1033–46.

199. Sheng Sow H, Mattarollo SR. Combining low-dose or metronomic chemotherapy with anticancer vaccines: A therapeutic opportunity for lymphomas. *Oncoimmunology*. 2013;2(12):e27058.

200. Youn J-I, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol.* 2008;181(8):5791–5802.

201. Ramachandran IR, Martner A, Pisklakova A, et al. Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow. *J. Immunol.* 2013;190(7):3815–23.

202. Van Valckenborgh E, Schouppe E, Movahedi K, et al. Multiple myeloma induces the immunosuppressive capacity of distinct myeloid-derived suppressor cell subpopulations in the bone marrow. *Leukemia*. 2012;

203. Haile LA, Gamrekelashvili J, Manns MP, Korangy F, Greten TF. CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice. *J. Immunol.* 2010;185(1):203–210.

204. Virtuoso LP, Harden JL, Sotomayor P, et al. Characterization of iNOS+ Neutrophil-like ring cell in tumor-bearing mice. *J. Transl. Med.* 2012;10(1):152.

205. Huang B, Pan PY, Li Q, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006;66(2):1123–1131.

206. Wu J, Zhang R, Tang N, et al. Dopamine inhibits the function of Gr-1+CD115+ myeloid-derived suppressor cells through D1-like receptors and enhances anti-tumor immunity. *J. Leukoc. Biol.* 2015;97(1):191–200.

207. Youn J-I, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *J. Leukoc. Biol.* 2012;91(1):167–181.

208. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J. Immunol.* 2005;175(7):4583–4592.

209. Movahedi K, Guilliams M, Van Den Bossche J, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell suppressive activity. *Blood*. 2008;111(8):4233–4244.

210. Cheng P, Corzo CA, Luetteke N, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J. Exp. Med.* 2008;205(10):2235–2249.

211. Sawanobori Y, Ueha S, Kurachi M, et al. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood*. 2008;111(12):5457–5466.

212. Dolcetti L, Peranzoni E, Ugel S, et al. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *Eur. J. Immunol.* 2010;40(1):22–35.

213. Nicholson LB, Raveney BJE, Munder M. Monocyte dependent regulation of autoimmune inflammation. *Curr. Mol. Med.* 2009;9(1):23–29.

214. Van Ginderachter JA, Beschin A, Baetselier P De, Raes G. Myeloid-derived suppressor cells in parasitic infections. *Eur. J. Immunol.* 2010;40(11):2976–2985.

215. Cripps JG, Gorham JD. MDSC in autoimmunity. *Int. Immunopharmacol.* 2011;11(7):789–793.

216. Chen X, Eksioglu EA, Zhou J, et al. Induction of myelodysplasia by myeloid-derived suppressor cells. *J. Clin. Invest.* 2013;123(11):4595–4611.

217. Solito S, Falisi E, Diaz-Montero CM, et al. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood*. 2011;118(8):2254–2265.

218. Youn J-I, Kumar V, Collazo M, et al. Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. *Nat. Immunol.* 2013;14(3):211–20.

219. De Vlaeminck Y, González-Rascón A, Goyvaerts C, Breckpot K. Cancer-Associated Myeloid Regulatory Cells. *Front. Immunol.* 2016;7:113.

220. Rodriguez PC, Ernstoff MS, Hernandez C, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res.* 2009;69(4):1553–1560.

221. S. George, P. Rayman, S. Biswas, T. Smith-Williams, J. S. Ko, L. Wood, P. Elson, B. I. Rini RMB and JHF. No Title. *ASCO*. 2008;

222. Mandruzzato S, Solito S, Falisi E, et al. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J. Immunol.* 2009;182(10):6562–6568.

223. Feng P-H, Lee K-Y, Chang Y-L, et al. CD14(+)S100A9(+) monocytic myeloid-derived suppressor cells and their clinical relevance in non-small cell lung cancer. *Am. J. Respir. Crit. Care Med.* 2012;186(10):1025–36.

224. Wang L, Chang EWY, Wong SC, et al. Increased myeloid-derived suppressor cells in gastric cancer correlate with cancer stage and plasma S100A8/A9 proinflammatory proteins. *J. Immunol.* 2013;190(2):794–804.

225. Zhao F, Hoechst B, Duffy A, et al. S100A9 a new marker for monocytic human myeloid-derived suppressor cells. *Immunology*. 2012;136(2):176–183.

226. Qin H, Lerman B, Sakamaki I, et al. Generation of a new therapeutic peptide that depletes myeloid-derived suppressor cells in tumor-bearing mice. *Nat. Med.* 2014;20(6):676–81.

227. Brandau S, Trellakis S, Bruderek K, et al. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *J. Leukoc. Biol.* 2011;89(2):311–317.

228. Heuvers ME, Muskens F, Bezemer K, et al. Arginase-1 mRNA expression correlates with myeloid-derived suppressor cell levels in peripheral blood of NSCLC patients. *Lung Cancer*. 2013;81(3):468–74.

229. Trellakis S, Bruderek K, Hütte J, et al. Granulocytic myeloid-derived suppressor

cells are cryosensitive and their frequency does not correlate with serum concentrations of colony-stimulating factors in head and neck cancer. *Innate Immun.* 2013;19:328–36.

230. Kotsakis A, Harasymczuk M, Schilling B, et al. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. *J. Immunol. Methods*. 2012;381(1–2):14–22.

231. Geissmann F, Manz MG, Jung S, et al. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010;327(5966):656–661.

232. Serafini P. Myeloid derived suppressor cells in physiological and pathological conditions: The good, the bad, and the ugly. *Immunol. Res.* 2013;57(1–3):172–184.

233. Porembka MR, Mitchem JB, Belt BA, et al. Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth. *Cancer Immunol. Immunother.* 2012;61(9):1373–1385.

234. Diaz-Montero CM, Salem ML, Nishimura MI, et al. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother.* 2009;58(1):49–59.

235. Salvadori S, Martinelli G, Zier K. Resection of solid tumors reverses T cell defects and restores protective immunity. *J. Immunol.* 2000;164(4):2214–2220.

236. Draghiciu O, Nijman HW, Hoogeboom BN, Meijerhof T, Daemen T. Sunitinib depletes myeloid-derived suppressor cells and synergizes with a cancer vaccine to enhance antigen-specific immune responses and tumor eradication. *Oncoimmunology*. 2015;4(3):e989764.

237. Filipazzi P, Valenti R, Huber V, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a

granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J. Clin. Oncol.* 2007;25(18):2546–2553.

238. Vuk-Pavlović S, Bulur PA, Lin Y, et al. Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. *Prostate*. 2010;70(4):443–455.

239. Hoechst B, Ormandy LA, Ballmaier M, et al. A New Population of Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Induces CD4+CD25+Foxp3+ T Cells. *Gastroenterology*. 2008;135(1):234–243.

240. Weed DT, Vella JL, Reis IM, et al. Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma. *Clin. Cancer Res.* 2015;21(1):39–48.

241. Aliper AM, Frieden-Korovkina VP, Buzdin A, Roumiantsev SA, Zhavoronkov A. Interactome analysis of myeloid-derived suppressor cells in murine models of colon and breast cancer. *Oncotarget*. 2014;5(22):11345–53.

242. Gielen PR, Schulte BM, Kers-Rebel ED, et al. Increase in both CD14-positive and CD15-positive myeloid-derived suppressor cell subpopulations in the blood of patients with glioma but predominance of CD15-positive myeloid-derived suppressor cells in glioma tissue. *J. Neuropathol. Exp. Neurol.* 2015;74(5):390–400.

243. Khaled YS, Ammori BJ, Elkord E. Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients. *J. Immunol. Res.* 2014;2014:879897.

244. Liu CY, Wang YM, Wang CL, et al. Population alterations of l-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14-/CD15+/CD33 + myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J. Cancer Res. Clin. Oncol.* 2010;136(1):35–45.

245. Lin Y, Gustafson MP, Bulur PA, et al. Immunosuppressive CD14+HLA-DRlow/-

monocytes in B-cell non-Hodgkin lymphoma. *Blood*. 2011;117(3):872–881.

246. Sato Y, Shimizu K, Shinga J, et al. Characterization of the myeloid-derived suppressor cell subset regulated by NK cells in malignant lymphoma. *Oncoimmunology*. 2015;4(3):e995541.

247. Romano A, Parrinello NL, Vetro C, et al. Circulating myeloid-derived suppressor cells correlate with clinical outcome in Hodgkin Lymphoma patients treated up-front with a risk-adapted strategy. *Br. J. Haematol.* 2015;168(5):689–700.

248. Liu J, Zhou Y, Huang Q, Qiu L. CD14(+)HLA-DR(low/-) expression: A novel prognostic factor in chronic lymphocytic leukemia. *Oncol. Lett.* 2015;9(3):1167–1172.

249. Jitschin R, Braun M, Büttner M, et al. CLL-cells induce IDOhi CD14+HLA-DRlo myeloid-derived suppressor cells that inhibit T-cell responses and promote TRegs. *Blood*. 2014;124(5):750–760.

250. De Veirman K, Van Ginderachter JA, Lub S, et al. Multiple myeloma induces Mcl-1 expression and survival of myeloid-derived suppressor cells. *Oncotarget*. 2015;6(12):10532–47.

251. Ğoŕgun GT, Whitehill G, Anderson JL, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood*. 2013;121(15):2975–2987.

252. Brimnes MK, Vangsted AJ, Knudsen LM, et al. Increased level of both CD4+FOXP3+ Regulatory t Cells and CD14+HLA-DR-/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand. J. Immunol.* 2010;72(6):540–547.

253. Görgün GT, Whitehill G, Anderson JL, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood*. 2013;121(15):2975–2987.

254. Christiansson L, Söderlund S, Svensson E, et al. Increased Level of Myeloid-Derived Suppressor Cells, Programmed Death Receptor Ligand 1/Programmed Death Receptor 1, and Soluble CD25 in Sokal High Risk Chronic Myeloid Leukemia. *PLoS One*. 2013;8(1):.

255. Al. MS et. Myeloid Leukemias Directly Suppress T Cell Proliferation Through STAT3 and Arginase Pathways. *ASH*. 2013;

256. Lechner MG, Megiel C, Russell SM, et al. Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. *J. Transl. Med.* 2011;9:90.

257. Dufait I, Schwarze JK, Liechtenstein T, et al. Ex vivo generation of myeloid-derived suppressor cells that model the tumor immunosuppressive environment in colorectal cancer. *Oncotarget*. 2015;6(14):12369–82.

258. Bayne LJ, Beatty GL, Jhala N, et al. Tumor-Derived Granulocyte-Macrophage Colony-Stimulating Factor Regulates Myeloid Inflammation and T Cell Immunity in Pancreatic Cancer. *Cancer Cell*. 2012;21(6):822–835.

259. Bronte V, Chappell DB, Apolloni E, et al. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. *J. Immunol.* 1999;162(10):5728–5737.

260. Morales JK, Kmieciak M, Knutson KL, Bear HD, Manjili MH. GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells. *Breast Cancer Res. Treat.* 2010;123(1):39–49.

261. Liechtenstein T, Perez-Janices N, Gato M, et al. A highly efficient tumor-infiltrating MDSC differentiation system for discovery of anti-neoplastic targets, which

circumvents the need for tumor establishment in mice. *Oncotarget*. 2014;5(17):7843–57.

262. Zhou Z, French DL, Ma G, et al. Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells. *Stem Cells*. 2010;28(3):620–32.

263. Waight JD, Hu Q, Miller A, Liu S, Abrams SI. Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. *PLoS One*. 2011;6(11):.

264. Pan PY, Wang GX, Yin B, et al. Reversion of immune tolerance in advanced malignancy: Modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. *Blood*. 2008;111(1):219–228.

265. Priceman SJ, Sung JL, Shaposhnik Z, et al. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: Combating tumor evasion of antiangiogenic therapy. *Blood*. 2010;115(7):1461–1471.

266. Chen M-F, Kuan F-C, Yen T-C, et al. IL-6-stimulated CD11b+ CD14+ HLA-DR- myeloid-derived suppressor cells, are associated with progression and poor prognosis in squamous cell carcinoma of the esophagus. *Oncotarget*. 2014;5(18):8716–28.

267. Elkabets M, Ribeiro VSG, Dinarello CA, et al. IL-1?? regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. *Eur. J. Immunol.* 2010;40(12):3347–3357.

268. Zhao X, Rong L, Zhao X, et al. TNF signaling drives myeloid-derived suppressor cell accumulation. *J. Clin. Invest.* 2012;122(11):4094–4104.

269. Villanueva MT. Microenvironment: small containers, important cargo. *Nat. Rev. Cancer*. 2014;14(12):764.

270. Balaj L, Atai NA, Chen W, et al. Heparin affinity purification of extracellular vesicles. *Sci. Rep.* 2015;5:10266.

271. Webber J, Yeung V, Clayton A. Extracellular vesicles as modulators of the cancer microenvironment. *Semin. Cell Dev. Biol.* 2015;40:27–34.

272. Xiang X, Poliakov A, Liu C, et al. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int. J. Cancer*. 2009;124(11):2621–2633.

273. Valenti R, Huber V, Filipazzi P, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res.* 2006;66(18):9290–8.

274. Rébé C, Végran F, Berger H, Ghiringhelli F. STAT3 activation: A key factor in tumor immunoescape. *JAK-STAT*. 2013;2(1):e23010.

275. Miner S, Ito S, Tanimoto K, Hensel N, Barrett J. Myeloid Leukemias directly suppress T cell proliferation through STAT3 and Arginase pathways. *ASH 2013*. 2013;(3885):.

276. Trikha P, Carson WE. Signaling pathways involved in MDSC regulation. *Biochim. Biophys. Acta - Rev. Cancer*. 2014;1846(1):55–65.

277. Dufait I, Van Valckenborgh E, Menu E, et al. Signal transducer and activator of transcription 3 in myeloid-derived suppressor cells: an opportunity for cancer therapy. *Oncotarget*. 2016;

278. Cohen PA, Ko JS, Storkus WJ, et al. Myeloid-derived suppressor cells adhere to physiologic STAT3- vs STAT5-dependent hematopoietic programming, establishing diverse tumor-mediated mechanisms of immunologic escape. *Immunol. Invest.* 2012;41(6–7):680–710.

279. Yu S, Liu C, Su K, et al. Tumor exosomes inhibit differentiation of bone marrow

dendritic cells. *J. Immunol.* 2007;178(11):6867–75.

280. Vasquez-Dunddel D, Pan F, Zeng Q, et al. STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. *J. Clin. Invest.* 2013;123(4):1580–9.

281. Kujawski M, Kortylewski M, Lee H, et al. Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice. *J. Clin. Invest.* 2008;118(10):3367–3377.

282. Xin H, Zhang C, Herrmann A, et al. Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells. *Cancer Res.* 2009;69(6):2506–2513.

283. Wu L, Du H, Li Y, Qu P, Yan C. Signal Transducer and Activator of Transcription 3 (Stat3C) Promotes Myeloid-Derived Suppressor Cell Expansion and Immune Suppression during Lung Tumorigenesis. *Am. J. Pathol.* 2011;179(4):2131–2141.

284. Mundy-Bosse BL, Young GS, Bauer T, et al. Distinct myeloid suppressor cell subsets correlate with plasma IL-6 and IL-10 and reduced interferon-alpha signaling in CD4 + T cells from patients with GI malignancy. *Cancer Immunol. Immunother.* 2011;60(9):1269–1279.

285. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J. Immunol.* 2005;174(2):636–645.

286. Wang X, Chang X, Zhuo G, Sun M, Yin K. Twist and miR-34a are involved in the generation of tumor-educated myeloid-derived suppressor cells. *Int. J. Mol. Sci.* 2013;14(10):20459–20477.

287. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer*. 2003;3(1):11–22.

288. Behre G, Singh SM, Liu H, et al. Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248. *J. Biol.*

Chem. 2002;277(29):26293–26299.

289. Clark CE, Hingorani SR, Mick R, et al. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res.* 2007;67(19):9518–9527.

290. Enioutina EY, Bareyan D, Daynes RA. A role for immature myeloid cells in immune senescence. *J. Immunol.* 2011;186(2):697–707.

291. Cully M, You H, Levine AJ, Mak TW. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat. Rev. Cancer*. 2006;6(3):184–192.

292. Ghansah T. A novel strategy for modulation of MDSC to enhance cancer immunotherapy. *Oncoimmunology*. 2012;1(6):984–985.

293. Li L, Zhang J, Diao W, et al. MicroRNA-155 and MicroRNA-21 promote the expansion of functional myeloid-derived suppressor cells. *J. Immunol.* 2014;192(3):1034–43.

294. Chen S, Wang L, Fan J, et al. Host miR155 promotes tumor growth through a myeloid-derived suppressor cell-dependent mechanism. *Cancer Res.* 2015;75(3):519–31.

295. Kim S, Song JH, Kim S, et al. Loss of oncogenic miR-155 in tumor cells promotes tumor growth by enhancing C/EBP-β-mediated MDSC infiltration. *Oncotarget*. 2016;7(10):11094–112.

296. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol. Sci.* 2012;33(4):207–14.

297. Zhao C, Zhang Q, Yu T, et al. Hypoxia promotes drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling. *J. bone Oncol.* 2016;5(1):22–9.

298. Syu J-P, Chi J-T, Kung H-N. Nrf2 is the key to chemotherapy resistance in MCF7 breast cancer cells under hypoxia. *Oncotarget*. 2016;

299. Rankin EB, Giaccia AJ. Hypoxic control of metastasis. *Science (80-.).* 2016;352(6282):175–180.

300. Sceneay J, Parker BS, Smyth MJ, Möller A. Hypoxia-driven immunosuppression contributes to the pre-metastatic niche. *Oncoimmunology*. 2013;2(1):e22355.

301. Duechler M, Peczek L, Zuk K, et al. The heterogeneous immune microenvironment in breast cancer is affected by hypoxia-related genes. *Immunobiology*. 2014;219(2):158–65.

302. Kumar V, Gabrilovich DI. Hypoxia-inducible factors in regulation of immune responses in tumour microenvironment. *Immunology*. 2014;143(4):512–9.

303. Corzo CA, Condamine T, Lu L, et al. HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J. Exp. Med.* 2010;207(11):2439–53.

304. Noman MZ, Janji B, Hu S, et al. Tumor-Promoting Effects of Myeloid-Derived Suppressor Cells Are Potentiated by Hypoxia-Induced Expression of miR-210. *Cancer Res.* 2015;75(18):3771–87.

305. Mikysková R, Indrová M, Vlková V, et al. DNA demethylating agent 5-azacytidine inhibits myeloid-derived suppressor cells induced by tumor growth and cyclophosphamide treatment. *J. Leukoc. Biol.* 2014;

306. Sevko A, Sade-Feldman M, Kanterman J, et al. Cyclophosphamide promotes chronic inflammation-dependent immunosuppression and prevents antitumor response in melanoma. *J. Invest. Dermatol.* 2013;133(6):1610–9.

307. Ding Z-C, Lu X, Yu M, et al. Immunosuppressive myeloid cells induced by chemotherapy attenuate antitumor CD4+ T-cell responses through the PD-1-PD-

L1 axis. *Cancer Res.* 2014;74(13):3441–53.

308. Dang Y, Wagner WM, Gad E, et al. Dendritic cell-activating vaccine adjuvants differ in the ability to elicit antitumor immunity due to an adjuvant-specific induction of immunosuppressive cells. *Clin. Cancer Res.* 2012;18(11):3122–31.

309. Bogdan C. Nitric oxide and the immune response. *Nat. Immunol.* 2001;2(10):907–916.

310. Wu G, Morris SM. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* 1998;336 (Pt 1:1–17.

311. Popovic PJ, Zeh HJ, Ochoa JB. Arginine and immunity. *J. Nutr.* 2007;137(6 Suppl 2):1681S–1686S.

312. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J. Immunol.* 1998;160(12):5729–5734.

313. Harari O, Liao JK. Inhibition of MHC II gene transcription by nitric oxide and antioxidants. *Curr. Pharm. Des.* 2004;10(8):893–898.

314. Mannick JB, Hausladen A, Liu L, et al. Fas-induced caspase denitrosylation. *Science*. 1999;284(5414):651–654.

315. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res.* 2001;61(12):4756–4760.

316. Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat. Med.* 2007;13(7):828–835.

317. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat. Rev. Immunol.* 2005;5(8):641–654.

318. Youn J-I, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur. J. Immunol.* 2010;40(11):2969–2975.

319. Mougiakakos D, Jitschin R, von Bahr L, et al. Immunosuppressive CD14+HLA-DRlow/neg IDO+ myeloid cells in patients following allogeneic hematopoietic stem cell transplantation. *Leukemia*. 2012;

320. Sakuishi K, Jayaraman P, Behar SM, Anderson AC, Kuchroo VK. Emerging Tim-3 functions in antimicrobial and tumor immunity. *Trends Immunol.* 2011;32(8):345–349.

321. Liechtenstein T, Perez-Janices N, Blanco-Luquin I, et al. Anti-melanoma vaccines engineered to simultaneously modulate cytokine priming and silence PD-L1 characterized using ex vivo myeloid-derived suppressor cells as a readout of therapeutic efficacy. *Oncoimmunology*. 3(7):e945378.

322. Chavan R, Salvador D, Gustafson MP, et al. Untreated stage IV melanoma patients exhibit abnormal monocyte phenotypes and decreased functional capacity. *Cancer Immunol. Res.* 2014;2(3):241–8.

323. Young MR, Wright MA, Matthews JP, Malik I, Prechel M. Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor-beta and nitric oxide. *J. Immunol.* 1996;156(5):1916–1922.

324. Terabe M, Matsui S, Park J-M, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J. Exp. Med.* 2003;198(11):1741–1752.

325. Liu Y, Lai L, Chen Q, et al. MicroRNA-494 is required for the accumulation and functions of tumor-expanded myeloid-derived suppressor cells via targeting of

PTEN. *J. Immunol.* 2012;188(11):5500–10.

326. Li H, Han Y, Guo Q, Zhang M, Cao X. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J. Immunol.* 2009;182(1):240–249.

327. Eruslanov E, Daurkin I, Ortiz J, Vieweg J, Kusmartsev S. Pivotal Advance: Tumor-mediated induction of myeloid-derived suppressor cells and M2-polarized macrophages by altering intracellular PGE_2 catabolism in myeloid cells. *J. Leukoc. Biol.* 2010;88(5):839–848.

328. Rodriguez PC, Hernandez CP, Quiceno D, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J. Exp. Med.* 2005;202(7):931–939.

329. Zhang Y, Liu Q, Zhang M, et al. Fas signal promotes lung cancer growth by recruiting myeloid-derived suppressor cells via cancer cell-derived PGE2. *J. Immunol.* 2009;182(6):3801–3808.

330. Tu S, Bhagat G, Cui G, et al. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell*. 2008;14(5):408–419.

331. Yu J, Wang Y, Yan F, et al. Noncanonical NF-κB Activation Mediates STAT3-Stimulated IDO Upregulation in Myeloid-Derived Suppressor Cells in Breast Cancer. *J. Immunol.* 2014;

332. Nagaraj S, Schrum AG, Cho H-I, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J. Immunol.* 2010;184(6):3106–3116.

333. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J. Immunol.* 2001;166(9):5398–5406.

334. Chalmin F, Ladoire S, Mignot G, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J. Clin. Invest.* 2010;120(2):457–471.

335. Kodumudi KN, Woan K, Gilvary DL, et al. A novel chemoimmunomodulating property of docetaxel: Suppression of myeloid-derived suppressor cells in tumor bearers. *Clin. Cancer Res.* 2010;16(18):4583–4594.

336. Serafini P, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res.* 2008;68(13):5439–5449.

337. Nagaraj S, Nelson A, Youn J -i., et al. Antigen-Specific CD4+ T Cells Regulate Function of Myeloid-Derived Suppressor Cells in Cancer via Retrograde MHC Class II Signaling. *Cancer Res.* 2012;72(4):928–938.

338. Chan LLY, Cheung BKW, Li JCB, Lau ASY. A role for STAT3 and cathepsin S in IL-10 down-regulation of IFN-gamma-induced MHC class II molecule on primary human blood macrophages. *J. Leukoc. Biol.* 2010;88(2):303–311.

339. Adeegbe D, Serafini P, Bronte V, et al. In Vivo Induction of Myeloid Suppressor Cells and CD4(+)Foxp3(+) T Regulatory Cells Prolongs Skin Allograft Survival in Mice. *Cell Transplant.* 2011;20(6):941–954.

340. Hoechst B, Gamrekelashvili J, Manns MP, Greten TF, Korangy F. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood*. 2011;117(24):6532–6541.

341. Pan PY, Ma G, Weber KJ, et al. Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res.* 2010;70(1):99–108.

342. Hoechst B, Voigtlaender T, Ormandy L, et al. Myeloid derived suppressor cells

inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology*. 2009;50(3):799–807.

343. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res.* 2007;67(9):4507–4513.

344. Narita Y, Wakita D, Ohkur T, Chamoto K, Nishimura T. Potential differentiation of tumor bearing mouse CD11b+Gr-1+ immature myeloid cells into both suppressor macrophages and immunostimulatory dendritic cells. *Biomed. Res.* 2009;30(1):7–15.

345. Hu C-E, Gan J, Zhang R-D, Cheng Y-R, Huang G-J. Up-regulated myeloid-derived suppressor cell contributes to hepatocellular carcinoma development by impairing dendritic cell function. *Scand. J. Gastroenterol.* 2011;46(2):156–164.

346. Poschke I, Mao Y, Adamson L, et al. Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines. *Cancer Immunol. Immunother.* 2012;61(6):827–838.

347. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J. Immunol.* 2007;179(2):977–983.

348. Kumar V, Cheng P, Condamine T, et al. CD45 Phosphatase Inhibits STAT3 Transcription Factor Activity in Myeloid Cells and Promotes Tumor-Associated Macrophage Differentiation. *Immunity*. 2016;44(2):303–15.

349. Xu J, Escamilla J, Mok S, et al. CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer Res.* 2013;73(9):2782–94.

350. Zhu Y, Knolhoff BL, Meyer MA, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint

immunotherapy in pancreatic cancer models. *Cancer Res.* 2014;74(18):5057–69.

351. Fujita M, Kohanbash G, Fellows-Mayle W, et al. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res.* 2011;71(7):2664–74.

352. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res.* 2011;71(24):7463–70.

353. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b + myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin. Cancer Res.* 2005;11(18):6713–6721.

354. Ko HJ, Kim YJ, Kim YS, et al. A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. *Cancer Res.* 2007;67(15):7477–7486.

355. Kusmartsev S, Cheng F, Yu B, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res.* 2003;63(15):4441–4449.

356. Bose A, Taylor JL, Alber S, et al. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. *Int. J. Cancer*. 2011;129(9):2158–2170.

357. Ozao-Choy J, Ge M, Kao J, et al. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res.* 2009;69(6):2514–2522.

358. Maus M V., Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood*. 2014;123(17):2625–2635.

359. Highfill S, Smith J, Long A, et al. Neutralization of murine myeloid suppressor cells enhances the efficacy of GD2-specific CAR T cells directed against human sarcoma in a xenograft model (VAC11P.1010). *J. Immunol.* 2014;192(1 Supplement):205.11.

360. Burga RA, Thorn M, Point GR, et al. Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. *Cancer Immunol. Immunother.* 2015;64(7):817–29.

361. John LB, Devaud C, Duong CPM, et al. Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. *Clin. Cancer Res.* 2013;19(20):5636–5646.

362. Dahan R, Sega E, Engelhardt J, et al. FcγRs Modulate the Anti-tumor Activity of Antibodies Targeting the PD-1/PD-L1 Axis. *Cancer Cell*. 2015;28(3):285–95.

363. Bill MA, Fuchs JR, Li C, et al. The small molecule curcumin analog FLLL32 induces apoptosis in melanoma cells via STAT3 inhibition and retains the cellular response to cytokines with anti-tumor activity. *Mol. Cancer*. 2010;9:165.

364. Lu P, Yu B, Xu J. Cucurbitacin B Regulates Immature Myeloid Cell Differentiation and Enhances Antitumor Immunity in Patients with Lung Cancer. *Cancer Biother. Radiopharm.* 2012;27(8):495–503.

365. Tu SP, Jin H, Shi JD, et al. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. *Cancer Prev. Res. (Phila).* 2012;5(2):205–15.

366. Ko JS, Rayman P, Ireland J, et al. Direct and differential suppression of myeloid-derived suppressor cell subsets by sunitinib is compartmentally constrained. *Cancer Res.* 2010;70(9):3526–3536.

367. Kodera Y, Katanasaka Y, Kitamura Y, et al. Sunitinib inhibits lymphatic endothelial cell functions and lymph node metastasis in a breast cancer model through

inhibition of vascular endothelial growth factor receptor 3. *Breast Cancer Res.* 2011;13(3):R66.

368. Finke JH, Rini B, Ireland J, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. *Clin. Cancer Res.* 2008;14(20):6674–6682.

369. Raymond E, Dalgleish A, Damber JE, Smith M, Pili R. Mechanisms of action of tasquinimod on the tumour microenvironment. *Cancer Chemother. Pharmacol.* 2014;73(1):1–8.

370. Schilling B, Sucker A, Griewank K, et al. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells. *Int. J. Cancer*. 2013;133(7):1653–1663.

371. Mirza N, Fishman M, Fricke I, et al. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res.* 2006;66(18):9299–9307.

372. Nefedova Y, Fishman M, Sherman S, et al. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res.* 2007;67(22):11021–11028.

373. Waldron TJ, Quatromoni JG, Karakasheva T a, Singhal S, Rustgi AK. Myeloid derived suppressor cells: Targets for therapy. *Oncoimmunology*. 2013;2(4):e24117.

374. Testa U, Masciulli R, Tritarelli E, et al. Transforming growth factor-beta potentiates vitamin D3-induced terminal monocytic differentiation of human leukemic cell lines. *J. Immunol.* 1993;150(6):2418–2430.

375. Zhou J, Wu J, Chen X, et al. Icariin and its derivative, ICT, exert anti-inflammatory, anti-tumor effects, and modulate myeloid derived suppressive cells (MDSCs) functions. *Int. Immunopharmacol.* 2011;11(7):887–895.

376. Wu H, Tao N, Liu X, et al. Polysaccharide from Lentinus edodes Inhibits the Immunosuppressive Function of Myeloid-Derived Suppressor Cells. *PLoS One*. 2012;7(12):.

377. Kusmartsev S, Eruslanov E, Kübler H, et al. Oxidative stress regulates expression of VEGFR1 in myeloid cells: link to tumor-induced immune suppression in renal cell carcinoma. *J. Immunol.* 2008;181(1):346–353.

378. Roelofs AJ, Ebetino FH, Reszka AA, Russell RGG, Rogers MJ. Bisphosphonates: Mechanisms of Action. *Princ. Bone Biol. Two-Volume Set*. 2008;2:1737–1767.

379. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of Kit-ligand. *Cell*. 2002;109(5):625–637.

380. Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. *Cancer Res.* 2007;67(23):11438–11446.

381. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J. Exp. Med.* 2006;203(12):2691–2702.

382. De Santo C, Serafini P, Marigo I, et al. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc. Natl. Acad. Sci. U. S. A.* 2005;102(11):4185–4190.

383. Gao P, Zhang H, Dinavahi R, et al. HIF-Dependent Antitumorigenic Effect of Antioxidants In Vivo. *Cancer Cell*. 2007;12(3):230–238.

384. Shirota Y, Shirota H, Klinman DM. Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells. *J. Immunol.* 2012;188(4):1592–9.

385. Heckelsmiller K, Rall K, Beck S, et al. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.* 2002;169(7):3892–3899.

386. Kawarada Y, Ganss R, Garbi N, et al. NK- and CD8(+) T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. *J. Immunol.* 2001;167(9):5247–5253.

387. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat. Rev. Cancer*. 2007;7(5):357–369.

388. Konopleva M, Zhang W, Shi Y-X, et al. Synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest in HER2-overexpressing breast cancer cells. *Mol. Cancer Ther.* 2006;5(2):317–328.

389. Muralikrishnan G, Dinda AK, Shakeel F. Immunomodulatory effects of Withania somnifera on azoxymethane induced experimental colon cancer in mice. *Immunol. Invest.* 2010;39(7):688–698.

390. Sinha P, Ostrand-Rosenberg S. Myeloid-derived suppressor cell function is reduced by Withaferin A, a potent and abundant component of Withania somnifera root extract. *Cancer Immunol. Immunother.* 2013;62(11):1663–1673.

391. Seung LP, Rowley DA, Dubey P, Schreiber H. Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection. *Proc. Natl. Acad. Sci. U. S. A.* 1995;92(14):6254–6258.

392. Roth F, De La Fuente AC, Vella JL, et al. Aptamer-mediated blockade of IL4R?? triggers apoptosis of MDSCs and limits tumor progression. *Cancer Res.* 2012;72(6):1373–1383.

393. Gallina G, Dolcetti L, Serafini P, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J. Clin. Invest.*

2006;116(10):2777–2790.

394. Le HK, Graham L, Cha E, et al. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int. Immunopharmacol.* 2009;9(7–8):900–909.

395. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* 2010;70(8):3052–3061.

396. Bruchard M, Mignot G, Derangère V, et al. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat. Med.* 2013;19(1):57–64.

397. Amato RJ, Hawkins RE, Kaufman HL, et al. Vaccination of metastatic renal cancer patients with MVA-5T4: a randomized, double-blind, placebo-controlled phase III study. *Clin. Cancer Res.* 2010;16(22):5539–5547.

398. Iclozan C, Antonia S, Chiappori A, Chen D-T, Gabrilovich D. Therapeutic regulation of myeloid-derived suppressor cells and immune response to cancer vaccine in patients with extensive stage small cell lung cancer. *Cancer Immunol. Immunother.* 2013;62(5):909–18.

399. clinicaltrials.gov. 2015;

400. Khan IN, Al-Karim S, Bora RS, Chaudhary AG, Saini KS. Cancer stem cells: a challenging paradigm for designing targeted drug therapies. *Drug Discov. Today*. 2015;

401. Grosicki S, Holowiecki J, Kuliczkowski K, et al. Assessing the efficacy of allogeneic hematopoietic stem cells transplantation (allo-HSCT) by analyzing survival end points in defined groups of acute myeloid leukemia patients; a retrospective, multicenter Polish Adult Leukemia Group study. *Am. J. Hematol.* 2015;

402. Odero MD, Zeleznik-Le NJ, Chinwalla V, Rowley JD. Cytogenetic and molecular analysis of the acute monocytic leukemia cell line THP-1 with anMLL-AF9 translocation. *Genes, Chromosom. Cancer*. 2000;29(4):333–338.

403. Matsuo Y, MacLeod RA, Uphoff CC, et al. Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). *Leukemia*. 1997;11(9):1469–77.

404. Verschoor CP, Johnstone J, Millar J, et al. Blood CD33(+)HLA-DR(-) myeloid-derived suppressor cells are increased with age and a history of cancer. *J. Leukoc. Biol.* 2013;93(4):633–7.

405. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506(7488):328–33.

406. Rechavi O, Erlich Y, Amram H, et al. Cell contact-dependent acquisition of cellular and viral nonautonomously encoded small RNAs. *Genes Dev.* 2009;23(16):1971–9.

407. Zomer A, Vendrig T, Hopmans ES, et al. Exosomes: Fit to deliver small RNA. *Commun. Integr. Biol.* 2010;3(5):447–50.

408. Di Vizio D, Kim J, Hager MH, et al. Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res.* 2009;69(13):5601–9.

409. Zhang J, Li S, Li L, et al. Exosome and Exosomal MicroRNA: Export, Sorting, and Function. *Genomics. Proteomics Bioinformatics*. 2015;13(1):17–24.

410. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2002;2(8):569–79.

411. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 2013;200(4):373–83.

412. Hong CS, Muller L, Boyiadzis M, Whiteside TL. Isolation and characterization of CD34+ blast-derived exosomes in acute myeloid leukemia. *PLoS One*. 2014;9(8):e103310.

413. Perez-Hernandez D, Gutiérrez-Vázquez C, Jorge I, et al. The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes. *J. Biol. Chem.* 2013;288(17):11649–61.

414. Tzoran I, Rebibo-Sabbah A, Brenner B, Aharon A. Disease dynamics in patients with acute myeloid leukemia: New biomarkers. *Exp. Hematol.* 2015;43(11):936–43.

415. Hornick NI, Huan J, Doron B, et al. Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. *Sci. Rep.* 2015;5:11295.

416. Huan J, Hornick NI, Shurtleff MJ, et al. RNA export by acute myelogenous leukemia exosomes. *Cancer Res.* 2013;73(2):918–29.

417. Valadi H, Ekström K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 2007;9(6):654–9.

418. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat. Cell Biol.* 2009;11(9):1143–9.

419. Crescitelli R, Lässer C, Szabo TG, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J. Extracell. Vesicles*. 2013;2:.

420. Ekström K, Valadi H, Sjöstrand M, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *J. Extracell. Vesicles*. 2012;1:.

421. Chevillet JR, Kang Q, Ruf IK, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl. Acad. Sci. U. S. A.* 2014;111(41):14888–93.

422. Stevanato L, Thanabalasundaram L, Vysokov N, Sinden JD. Investigation of Content, Stoichiometry and Transfer of miRNA from Human Neural Stem Cell Line Derived Exosomes. *PLoS One*. 2016;11(1):e0146353.

423. Blanc L, De Gassart A, Géminard C, Bette-Bobillo P, Vidal M. Exosome release by reticulocytes--an integral part of the red blood cell differentiation system. *Blood Cells. Mol. Dis.* 35(1):21–6.

424. Meckes DG, Raab-Traub N. Microvesicles and viral infection. *J. Virol.* 2011;85(24):12844–54.

425. André F, Chaput N, Schartz NEC, et al. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J. Immunol.* 2004;172(4):2126–36.

426. Vincent-Schneider H, Stumptner-Cuvelette P, Lankar D, et al. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int. Immunol.* 2002;14(7):713–22.

427. Théry C, Duban L, Segura E, et al. Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* 2002;3(12):1156–62.

428. Ristorcelli E, Beraud E, Verrando P, et al. Human tumor nanoparticles induce apoptosis of pancreatic cancer cells. *FASEB J.* 2008;22(9):3358–69.

429. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 2008;10(12):1470–6.

430. Qu J-L, Qu X-J, Zhao M-F, et al. Gastric cancer exosomes promote tumour cell

proliferation through PI3K/Akt and MAPK/ERK activation. *Dig. Liver Dis.* 2009;41(12):875–880.

431. Park JE, Tan H Sen, Datta A, et al. Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. *Mol. Cell. Proteomics*. 2010;9(6):1085–99.

432. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat. Rev. Immunol.* 2014;14(3):195–208.

433. Liu C, Yu S, Kappes J, et al. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood*. 2007;109(10):4336–42.

434. Liu Y, Xiang X, Zhuang X, et al. Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. *Am. J. Pathol.* 2010;176(5):2490–9.

435. Xiang X, Liu Y, Zhuang X, et al. TLR2-mediated expansion of MDSCs is dependent on the source of tumor exosomes. *Am. J. Pathol.* 2010;177(4):1606–10.

436. Szczepanski MJ, Szajnik M, Welsh A, Whiteside TL, Boyiadzis M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. *Haematologica*. 2011;96(9):1302–9.

437. Hong CS, Muller L, Boyiadzis M, Whiteside TL. Isolation and characterization of CD34+ blast-derived exosomes in acute myeloid leukemia. *PLoS One*. 2014;9(8):e103310.

438. Whiteside TL. Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumour-derived exosomes). *Biochem. Soc. Trans.* 2013;41(1):245–51.

439. Ohyashiki JH, Umezu T, Ohyashiki K. Exosomes promote bone marrow angiogenesis in hematologic neoplasia: the role of hypoxia. *Curr. Opin. Hematol.*

2016;

440. Wojtuszkiewicz A, Schuurhuis GJ, Kessler FL, et al. Exosomes secreted by apoptosis-resistant AML blasts harbor regulatory network proteins potentially involved in antagonism of apoptosis. *Mol. Cell. Proteomics*. 2016;

441. Kalinkovich A, Tavor S, Avigdor A, et al. Functional CXCR4-expressing microparticles and SDF-1 correlate with circulating acute myelogenous leukemia cells. *Cancer Res.* 2006;66(22):11013–20.

442. Ford T, Graham J, Rickwood D. Iodixanol: a nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. *Anal. Biochem.* 1994;220(2):360–6.

443. Vlassov A V, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim. Biophys. Acta*. 2012;1820(7):940–8.

444. Peterson MF, Otoc N, Sethi JK, Gupta A, Antes TJ. Integrated systems for exosome investigation. *Methods*. 2015;87:31–45.

445. Shin H, Han C, Labuz JM, et al. High-yield isolation of extracellular vesicles using aqueous two-phase system. *Sci. Rep.* 2015;5:13103.

446. Enderle D, Spiel A, Coticchia CM, et al. Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method. *PLoS One*. 2015;10(8):e0136133.

447. Oksvold MP, Neurauter A, Pedersen KW. Magnetic bead-based isolation of exosomes. *Methods Mol. Biol.* 2015;1218:465–81.

448. VAN DER POL E, HOEKSTRA AG, STURK A, et al. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *J. Thromb. Haemost.* 2010;8(12):2596–2607.

449. Wright M. Nanoparticle tracking analysis for the multiparameter characterization and counting of nanoparticle suspensions. *Methods Mol. Biol.* 2012;906:511–24.

450. Soo CY, Song Y, Zheng Y, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology*. 2012;136(2):192–7.

451. Erdbrügger U, Lannigan J. Analytical challenges of extracellular vesicle detection: A comparison of different techniques. *Cytometry. A*. 2016;89(2):123–34.

452. Nolte-'t Hoen ENM, van der Vlist EJ, Aalberts M, et al. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. *Nanomedicine*. 2012;8(5):712–20.

453. Groot Kormelink T, Arkesteijn GJA, Nauwelaers FA, et al. Prerequisites for the analysis and sorting of extracellular vesicle subpopulations by high-resolution flow cytometry. *Cytometry. A*. 2016;89(2):135–47.

454. El-Andaloussi S, Lee Y, Lakhal-Littleton S, et al. Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat. Protoc.* 2012;7(12):2112–26.

455. Pospichalova V, Svoboda J, Dave Z, et al. Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *J. Extracell. Vesicles*. 2015;4:.

456. Campanella C, Rappa F, Sciumè C, et al. Heat shock protein 60 levels in tissue and circulating exosomes in human large bowel cancer before and after ablative surgery. *Cancer*. 2015;121(18):3230–9.

457. Hegmans JPJJ, Bard MPL, Hemmes A, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am. J. Pathol.* 2004;164(5):1807–15.

458. Roberg-Larsen H, Lund K, Seterdal KE, et al. Mass spectrometric detection of 27-hydroxycholesterol in breast cancer exosomes. *J. Steroid Biochem. Mol. Biol.*

2016;

459. Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* 2005;17(7):879–87.

460. Lekchnov EA, Zaporozhchenko IA, Morozkin ES, et al. Protocol for miRNA isolation from biofluids. *Anal. Biochem.* 2016;499:78–84.

461. El-Khoury V, Pierson S, Kaoma T, Bernardin F, Berchem G. Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Sci. Rep.* 2016;6:19529.

462. Delić D, Eisele C, Schmid R, et al. Urinary Exosomal miRNA Signature in Type II Diabetic Nephropathy Patients. *PLoS One*. 2016;11(3):e0150154.

463. Bretz NP, Ridinger J, Rupp A-K, et al. Body fluid exosomes promote secretion of inflammatory cytokines in monocytic cells via Toll-like receptor signaling. *J. Biol. Chem.* 2013;288(51):36691–702.

464. Sivadasan P, Gupta MK, Sathe GJ, et al. Human salivary proteome--a resource of potential biomarkers for oral cancer. *J. Proteomics*. 2015;127(Pt A):89–95.

465. Grigor'eva AE, Tamkovich SN, Eremina A V, et al. [Characteristics of exosomes andmicroparticles discovered in human tears]. *Biomed. Khim.* 2016;62(1):99–106.

466. Oksvold MP, Kullmann A, Forfang L, et al. Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. *Clin. Ther.* 2014;36(6):847–862.e1.

467. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* 2008;110(1):13–21.

468. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin. Lung Cancer*. 2009;10(1):42–6.

469. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 2008;18(10):997–1006.

470. Bryant RJ, Pawlowski T, Catto JWF, et al. Changes in circulating microRNA levels associated with prostate cancer. *Br. J. Cancer*. 2012;106(4):768–74.

471. Manier S, Avet-Loiseau H, Campigotto F, et al. Prognostic Value of Circulating Exosomal microRNAs in 112 Patients with Multiple Myeloma. *Blood*. 2014;124(21):2056.

472. Hornick NI, Huan J, Doron B, et al. Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. *Sci. Rep.* 2015;5:11295.

473. Takeshita N, Hoshino I, Mori M, et al. Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. *Br. J. Cancer*. 2013;108(3):644–52.

474. Viaud S, Théry C, Ploix S, et al. Dendritic cell-derived exosomes for cancer immunotherapy: what's next? *Cancer Res.* 2010;70(4):1281–5.

475. Dai S, Wei D, Wu Z, et al. Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol. Ther.* 2008;16(4):782–90.

476. Morse MA, Garst J, Osada T, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* 2005;3(1):9.

477. Escudier B, Dorval T, Chaput N, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J. Transl. Med.* 2005;3(1):10.

478. Akao Y, Iio A, Itoh T, et al. Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages. *Mol. Ther.* 2011;19(2):395–9.

479. Ohno S, Takanashi M, Sudo K, et al. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol. Ther.* 2013;21(1):185–91.

480. Alvarez-Erviti L, Seow Y, Yin H, et al. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* 2011;29(4):341–5.

481. Mizrak A, Bolukbasi MF, Ozdener GB, et al. Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth. *Mol. Ther.* 2013;21(1):101–8.

482. Tian Y, Li S, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials*. 2014;35(7):2383–90.

483. Kooijmans SAA, Vader P, van Dommelen SM, van Solinge WW, Schiffelers RM. Exosome mimetics: a novel class of drug delivery systems. *Int. J. Nanomedicine*. 2012;7:1525–41.

484. Sun D, Zhuang X, Xiang X, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol. Ther.* 2010;18(9):1606–14.

485. Hung ME, Leonard JN. Stabilization of exosome-targeting peptides via engineered glycosylation. *J. Biol. Chem.* 2015;290(13):8166–72.

486. Marleau AM, Chen C-S, Joyce JA, Tullis RH. Exosome removal as a therapeutic adjuvant in cancer. *J. Transl. Med.* 2012;10:134.

487. Gusnard D, Kirschner RH. Cell and organelle shrinkage during preparation for scanning electron microscopy: effects of fixation, dehydration and critical point drying. *J. Microsc.* 1977;110(1):51–7.

488. van der Pol E, Coumans FAW, Grootemaat AE, et al. Particle size distribution of

exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J. Thromb. Haemost.* 2014;12(7):1182–92.

489. Bouillez A, Rajabi H, Pitroda S, et al. Inhibition of MUC1-C Suppresses MYC Expression and Attenuates Malignant Growth in KRAS Mutant Lung Adenocarcinomas. *Cancer Res.* 2016;76(6):1538–48.

490. Tagde A, Rajabi H, Bouillez A, et al. MUC1-C drives MYC in multiple myeloma. *Blood*. 2016;127(21):2587–97.

491. Tagde A, Rajabi H, Stroopinsky D, et al. MUC1-C induces DNA methyltransferase 1 and represses tumor suppressor genes in acute myeloid leukemia. *Oncotarget*. 2016;

492. Yin L, Kosugi M, Kufe D. Inhibition of the MUC1-C oncoprotein induces multiple myeloma cell death by down-regulating TIGAR expression and depleting NADPH. *Blood*. 2012;119(3):810–6.

493. David JM, Hamilton DH, Palena C. MUC1 upregulation promotes immune resistance in tumor cells undergoing brachyury-mediated epithelial-mesenchymal transition. *Oncoimmunology*. 2016;5(4):e1117738.

494. Eilers M, Picard D, Yamamoto KR, Bishop JM. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature*. 1989;340(6228):66–8.

495. Tagde A, Rajabi H, Bouillez A, et al. MUC1-C drives MYC in multiple myeloma. *Blood*. 2016;

496. Bouillez A, Rajabi H, Pitroda S, et al. Inhibition of MUC1-C Suppresses MYC Expression and Attenuates Malignant Growth in KRAS Mutant Lung Adenocarcinomas. *Cancer Res.* 2016;76(6):1538–48.

497. Hu W, Coller J. What comes first: translational repression or mRNA degradation? The deepening mystery of microRNA function. *Cell Res.* 2012;22(9):1322–4.

498. Sun M, Chen H, Liu J, Tong C, Meng T. MicroRNA-34a inhibits human trophoblast cell invasion by targeting MYC. *BMC Cell Biol.* 2015;16:21.

499. Rajabi H, Tagde A, Alam M, et al. DNA methylation by DNMT1 and DNMT3b methyltransferases is driven by the MUC1-C oncoprotein in human carcinoma cells. *Oncogene*. 2016;

500. Srivastava P, Paluch BE, Matsuzaki J, et al. Immunomodulatory action of SGI-110, a hypomethylating agent, in acute myeloid leukemia cells and xenografts. *Leuk. Res.* 2014;38(11):1332–1341.

501. Guan H, Xie L, Klapproth K, et al. Decitabine represses translocated MYC oncogene in Burkitt lymphoma. *J. Pathol.* 2013;229(5):775–83.

502. Ng GZ, Sutton P. The MUC1 mucin specifically inhibits activation of the NLRP3 inflammasome. *Genes Immun.* 2016;17(3):203–6.

503. Rajabi H, Tagde A, Alam M, et al. DNA methylation by DNMT1 and DNMT3b methyltransferases is driven by the MUC1-C oncoprotein in human carcinoma cells. *Oncogene*. 2016;

504. Tagde A, Rajabi H, Stroopinsky D, et al. MUC1-C induces DNA methyltransferase 1 and represses tumor suppressor genes in acute myeloid leukemia. *Oncotarget*. 2016;

505. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell*. 1988;55(5):917–24.

506. Gazon H, Belrose G, Terol M, et al. Impaired expression of DICER and some microRNAs in HBZ expressing cells from acute adult T-cell leukemia patients.

Oncotarget. 2016;

507. Triozzi PL, Aldrich W, Achberger S, et al. Differential eVects of low-dose decitabine on immune effector and suppressor responses in melanoma-bearing mice. *Cancer Immunol. Immunother.* 2012;61(9):1441–1450.

508. Momparler RL. Pharmacology of 5-Aza-2'-deoxycytidine (decitabine). *Semin. Hematol.* 2005;42(3 Suppl 2):S9-16.

509. Klemm F, Joyce JA. Microenvironmental regulation of therapeutic response in cancer. *Trends Cell Biol*. 2014;

510. Wang Z, Zhang L, Wang H, et al. Tumor-induced CD14(+)HLA-DR (-/low) myeloid-derived suppressor cells correlate with tumor progression and outcome of therapy in multiple myeloma patients. *Cancer Immunol Immunother*. 2014;

511. Laborde RR, Lin Y, Gustafson MP, Bulur PA, Dietz AB. Cancer Vaccines in the World of Immune Suppressive Monocytes (CD14(+)HLA-DR(lo/neg) Cells): The Gateway to Improved Responses. *Front Immunol*. 2014;5:147.

512. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 2009;9(3):162–174.

513. Greten TF, Manns MP, Korangy F. Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol*. 2011;11(7):802–807.

514. Baniyash M, Sade-Feldman M, Kanterman J. Chronic inflammation and cancer: suppressing the suppressors. *Cancer Immunol Immunother*. 2014;63(1):11–20.

515. Katoh H, Wang D, Daikoku T, et al. CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. *Cancer Cell*. 2013;24(5):631–644.

516. Vedell PT, Svenson KL, Churchill GA, et al. Stochastic variation of transcript abundance in C57BL/6J mice. *BMC Genomics*. 2011;12(1):167.

517. Health USNI of. Clinicaltrials.gov. -. 2013;

518. Li XJ, Ren ZJ, Tang JH. MicroRNA-34a: a potential therapeutic target in human cancer. *Cell Death Dis*. 2014;5:e1327.

519. Baek D, Villén J, Shin C, et al. The impact of microRNAs on protein output. *Nature*. 2008;455(7209):64–71.

520. Pulikkan JA, Peramangalam PS, Dengler V, et al. C/EBPα regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with CEBPA mutations. *Blood*. 2010;116(25):5638–5649.

521. Cifuentes D, Xue H, Taylor DW, et al. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science*. 2010;328(5986):1694–8.

522. Tagde A, Singh H, Kang MH, Reynolds CP. The glutathione synthesis inhibitor buthionine sulfoximine synergistically enhanced melphalan activity against preclinical models of multiple myeloma. *Blood Cancer J.* 2014;4:e229.

523. Yang Y, Wang L-L, Li Y-H, et al. Effect of CpG island methylation on microRNA expression in the k-562 cell line. *Biochem. Genet.* 2012;50(1–2):122–34.

524. Pan X, Wang R, Wang Z-X, et al. The potential role of miR-451 in cancer diagnosis, prognosis, and therapy. *Mol. Cancer Ther.* 2013;12(7):1153–62.

525. Villela D, Ramalho RF, Silva ART, et al. Differential DNA Methylation of MicroRNA Genes in Temporal Cortex from Alzheimer's Disease Individuals. *Neural Plast.* 2016;2016:2584940.